PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 06 October 2000 (06.10.00)	
International application No. PCT/US00/00179	**Applicant's or agent's file reference** 30434.4WO01
International filing date (day/month/year) 05 January 2000 (05.01.00)	**Priority date** (day/month/year) 05 January 1999 (05.01.99)

Applicant

NOBLE, Nancy, A. et al

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

04 August 2000 (04.08.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expirati nths from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland** Facsimile No.: (41-22) 740.14.35	Authorized officer Kiwa Mpay Telephone No.: (41-22) 338.83.38

Form PCT/IB/331 (July 1992)

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: SARALYNN MANDEL
MANDEL & ADRIANO
35 NO. ARROYO PARKWAY
SUITE 60
PASADENA CA 91103

PCT

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of Mailing *(day/month/year)*	16 JUL 2001
Applicant's or agent's file reference 30434.4WO01	IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority Date *(day/month/year)*
PCT/US00/00179	05 JANUARY 2000	05 JANUARY 1999

Applicant
UNIVERSITY OF UTAH

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	SEAN MCGARRY Jorge Budgers for
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/IPEA/416 (July 1992)★

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 30434.4WO01	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US00/00179	International filing date *(day/month/year)* 05 JANUARY 2000	Priority date *(day/month/year)* 05 JANUARY 1999
International Patent Classification (IPC) or national classification and IPC IPC(7): A61K 31/00; A61P 13/12 and US Cl.: 514/2		
Applicant UNIVERSITY OF UTAH		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report

   II ☐ Priority

   III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability

   IV ☐ Lack of unity of invention

   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

   VI ☐ Certain documents cited

   VII ☐ Certain defects in the international application

   VIII ☐ Certain observations on the international application

Date of submission of the demand 04 AUGUST 2000	Date of completion of this report 26 JUNE 2001
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer SEAN MCGARRY Telephone No. (703) 308-0196

Form PCT/IPEA/409 (cover sheet) (July 1998)★

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US00/00179

I. Basis of the report

1. With regard to the **elements** of the international application:*

[X] the international application as originally filed

[X] the description:
pages 1-41 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the claims:
pages 42-48 , as originally filed
pages NONE , as amended (together with any statement) under Article 19
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the drawings:
pages 1-10 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the sequence listing part of the description:
pages NONE , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

[ ] contained in the international application in printed form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. [X] The amendments have resulted in the cancellation of:

[X] the description, pages NONE

[X] the claims, Nos. NONE

[X] the drawings, sheets/~~fig~~ NONE

5. [ ] This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

***Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	9-14, 20-25, 37-48	YES
	Claims	1-8, 15-19, 26-36, 49-51	NO
Inventive Step (IS)	Claims	9-14, 20-25, 37-48	YES
	Claims	1-8, 15-19, 26-36, 49-51	NO
Industrial Applicability (IA)	Claims	1-51	YES
	Claims	NONE	NO

2. citations and explanations (Rule 70.7)

Claims 1-8, 15-19, 26-36, 49-51 lack novelty under PCT Article 33(2) as being anticipated by WO 96 25178, WO 91 04748, Border et al. (Nature Vol. 360(6402: 361-364, 1992), Border et al. (Hypertension Vol. 31(1):181-188, 1998),and, Peters et al. (J. of The American Society of Nephrology Vol. 8: 524A, 1997).

WO 96 25178 discloses the treatment of various diseases such as glomerulonephritis, ARDS, cirrhosis, fibrosis, scarring, myocardial infarction via the administration to various tissues including kidney, liver, lung and skin a TGF-bets-specific inhibitory agent such as decorin, biglycan, fibromodulin, lumican and TGF-beta specific antibodies.

WO 91 04748 discloses prevention of accumulation of extracellular matrix via the inhibition of TGF-beta in tissues such as kidney, lung, liver and skin. It is disclosed treatment of various fibrotic diseases such as glomerulonephritis in diabetic nephropathy,and inhibitors such as TGF-beta specific antibodies, PDGF and Arg-Gly-Asp containing peptides.

Border et al (Nature) disclose inhibition of TGF-beta in treating scarring in kidney disease via the proteoglycan decorin and via antibodies to TGF-beta antibodies.

Border et al disclose the inhibition of TGF-beta via angiotensin II in renal fibrosis.

Peters et al disclose therapeutic inhibition of TGF-beta via angiotensin blockade via ace inhibitor enalapril.

Applicant has argued in the response filed 30 May 2001 that the references do not teach a combination of a TGF-beta inhibitor and agent that decreases ECM in the treatment or compositions claimed. It is noted that the claims at hand do embrace such combinations but do not specifically require such combinations.

Claims 9-14, 20-25, 37-48 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest a method of treating TGF-beta associated disorders via a combination therapy including the administration of a TGF-beta specific inhibitor as well as a different agent that degrades ECM (Continued on Supplemental Sheet.)

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):
proteins present in a diseased tissue.

---------- NEW CITATIONS ----------
NONE

09/869820

PATENT COOPERATION TREATY

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

14

Applicant's or agent's file reference 30434.4WO01	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US00/00179	International filing date *(day/month/year)* 05 JANUARY 2000	Priority date *(day/month/year)* 05 JANUARY 1999
International Patent Classification (IPC) or national classification and IPC IPC(7): A61K 31/00; A61P 13/12 and US Cl.: 514/2		
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Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer SEAN MCGARRY
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/IPEA/409 (cover sheet) (July 1998)★

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US00/00171

I. Basis of the report

1. With regard to the **elements** of the international application:*

[X] the international application as originally filed

[X] the description:
pages 1-41 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the claims:
pages 42-48 , as originally filed
pages NONE , as amended (together with any statement) under Article 19
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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US00/00179

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Continuation of: **Boxes I - VIII**

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):
proteins present in a diseased tissue.

---------- NEW CITATIONS ----------
NONE

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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : A61K 31/00

A2

(11) International Publication Number: WO 00/40227

(43) International Publication Date: 13 July 2000 (13.07.00)

(21) International Application Number: PCT/US00/00179

(22) International Filing Date: 5 January 2000 (05.01.00)

(30) Priority Data:
60/114,795 5 January 1999 (05.01.99) US

(71) Applicants *(for all designated States except US)*: UNIVERSITY OF UTAH [US/US]; 615 Arapeen Drive, Suite 110, Salt Lake City, UT 84108 (US). AMERICAN NATIONAL RED CROSS [US/US]; 15601 Crabbs Branch Way, Rockville, MD 20855 (US).

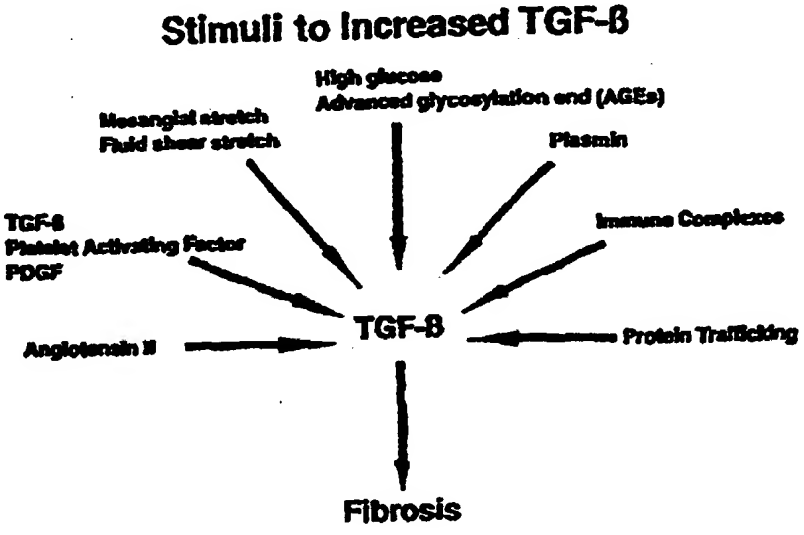
(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: NOBLE, Nancy, A. [US/US]; 4464 South Abinadi, Salt Lake City, UT 84124 (US). BORDER, Wayne, A. [US/US]; 4464 South Abinadi, Salt Lake City, UT 84124 (US). LAWRENCE, Daniel, A. [US/US]; 16525 Keats Terrace, Derwood, MD 20855 (US).

(74) Agent: MANDEL, Sara Lynn; Mandel & Adriano, 35 No. Arroyo Parkway, Suite 60, Pasadena, CA 91103 (US).

(81) Designated States: AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: METHODS FOR TREATING CONDITIONS ASSOCIATED WITH THE ACCUMULATION OF EXCESS EXTRACELLULAR MATRIX



(57) Abstract

The present invention is methods and compositions for reducing and preventing the excess accumulation of extracellular matrix in a tissue and/or organ or at a wound site using a combination of agents that inhibit TGFβ, or using agents that inhibit TGFβ in combination with agents that degrade excess accumulated extracellular matrix. The compositions and methods of the invention are used to treat conditions such as fibrotic diseases and scarring that result from excess accumulation of extracellular matrix, impairing tissue or organ function or skin appearance in a suject.

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METHODS FOR TREATING CONDITIONS ASSOCIATED WITH THE ACCUMULATION OF EXCESS EXTRACELLULAR MATRIX

By, Nancy A. Noble, Wayne A. Border and Daniel A. Lawrence

FIELD OF THE INVENTION

This invention relates to a method for preventing or reducing excess accumulation of extracellular matrix in tissues or organs or at a wound site, and more particularly to the prevention and treatment of conditions resulting from excess accumulation of extracellular matrix, using a combination of agents that inhibit TGFβ, or a combination of agents that inhibit TGFβ and agents that degrade excess accumulated extracellular matrix.

BACKGROUND OF THE INVENTION

Excess deposition and accumulation of extracellular matrix (ECM) is found in diseases such as fibrosis of the kidney or lung. Although the cytokine transforming growth factor Beta (TGFβ) regulates extracellular matrix deposition for tissue repair, overproduction of TGFβ clearly underlies tissue fibrosis caused by excess deposition of extracellular matrix resulting in disease (Border and Ruoslahti, *J. Clin. Invest.* 90:1-7 (1992)). TGFβ's fibrogenic action results from simultaneous stimulation of matrix protein synthesis (Border et al., *Kidney Int* 37:689-695 (1990), inhibition of matrix degradation and turnover and enhanced cell-matrix interactions through modulation of integrin receptors that facilitate ECM assembly. Overproduction of TGFβ has been demonstrated in glomerulonephritis (Okuda et al., *J. Clin. Invest.* 86:453-462 (1990)), diabetic nephropathy and hypertensive glomerular injury and in related fibrotic disorders of the lung, liver, heart, arterial wall, skin, brain, joints and bone marrow (Border and Noble, *N. Eng. J. Med.* 331:1286-1292 (1994)). In addition to the kidney, blocking the action of TGFβ with an agent such as antibody or the proteoglycan decorin has been shown to be therapeutic in fibrosis and scarring of the skin, lung, central nervous system and arterial wall (Border and Noble, *Kidney Int.* 51:1388-1396 (1997)).

Suppression of the production of ECM and prevention of excess accumulation of mesangial matrix in glomeruli of glomerulonephritic rats has been demonstrated by intravenous

administration of neutralizing antibodies specific for TGFβ (Border et al., *Nature* 346:371-374 (1990)) or administration of purified decorin, a proteoglycan (Border et al., *Nature* 360:361-364 (1992)) and by introduction of nucleic acid encoding decorin, a TGFβ-inhibitory agent, into a rat model of acute mesangial glomerulonephritis (Isaka et al., *Nature Med.* 2:418-423 (1996)). Inhibition of TGFβ activity, using for example anti-TGFβ antibodies, has been shown to to disrupt TGFβ overproduction (Sharma et al., *Diabetes* 45:522-530 (1996)).

Dermal scarring following dermal injury results from excessive accumulation of fibrous tissue made up of collagen, fibronectin and proteoglycans at a wound site. Because the fibrous extracellular matrix lacks elasticity, scar tissue can impair essential tissue function as well as result in an undesirable cosmetic appearance. TGFβ is believed to induce the deposition of fibrous matrix at the wound site (Shah et al., *Lancet* 339:213-214 (1992)).

One explanation for persistent TGFβ overexpression in progressive fibrotic kidney disease is that repeated or multiple episodes of tissue injury, such as occurs in chronic diseases such as hypertension, diabetes or immune complex disease lead to continuous overproduction of TGFβ and extracellular matrix resulting in tissue fibrosis (See Border and Noble, *N. Eng. J. Med.* 331:1286-1292 (1994)). Another possible explanation for persistent TGFβ overexpression is the presence of a biologically complex interconnection between TGFβ and the renin-angiotensin system (RAS) in the kidney as part of an emergency system that responds to the threat of tissue injury as discussed further herein.

Renin is an aspartyl proteinase synthesized by juxtaglomerular kidney cells and mesangial cells in humans and rats. (Chansel et al., *Am. J. Physiol.* 252:F32-F38 (1987) and Dzau and Kreisberg, *J. Cardiovasc. Pharmacol.* 8(Suppl 10):S6-S10 (1986)). Renin plays a key role in the regulation of blood pressure and salt balance. Its major source in humans is the kidney where it is initially produced as preprorenin. Signal peptide processing and glycosylation are followed by secretion of prorenin and its enzymatically active form, mature renin. The active enzyme triggers a proteolytic cascade by cleaving angiotensinogen to generate angiotensin I, which is in turn converted to the vasoactive hormone angiotensin II by angiotensin converting enzyme ("ACE").

The sequence of the human renin gene is known (GenBank entry M26901). Recombinant human renin has been synthesized and expressed in various expression systems (Sielecki et al., *Science* 243:1346-1351 (1988), Mathews et al., *Protein Expression and Purification* 7:81-91 (1996)). Inhibitors of renin's enzymatic site are known (Rahuel et al., *J. Struct. Biol.* 107:227-236 (1991); Badasso et al., *J. Mol. Biol.* 223:447-453 (1992); and Dhanaraj et al., *Nature* 357:466-472 (1992)) including an orally active renin inhibitor in primates, Ro 42-5892 (Fischli et al., *Hypertension* 18:22-31 (1991)). Renin-binding proteins and a cell surface renin receptor on human mesangial cells have been identified (Campbell and Valentijn, *J. Hypertens.* 12:879-890 (1994), Nguyen et al., *Kidney Internat.* 50:1897-1903 (1996) and Sealey et al., *Amer. J. Hyper.* 9:491-502 (1996)).

The renin-angiotensin system (RAS) is a prototypical systemic endocrine network whose actions in the kidney and adrenal glands regulate blood pressure, intravascular volume and electrolyte balance. In contrast, TGFβ is considered to be a prototypical cytokine, a peptide signaling molecule whose multiple actions on cells are mediated in a local or paracrine manner. Recent data however, indicate that there is an intact RAS in many tissues whose actions are entirely paracrine and TGFβ has wide-ranging systemic (endocrine) effects. Moreover, RAS and TGFβ act at various points to regulate the actions of one another.

In a systemic response to an injury such as a wound, the RAS rapidly generates AII that acts by vasoconstriction to maintain blood pressure and later stimulates the secretion of aldosterone, resulting in an increase in intravascular volume. In the wound, TGFβ is rapidly released by degranulating platelets and causes a number of effects including: 1) autoinduction of the production of TGFβ by local cells to amplify biological effects; 2) chemoattraction of monocyte/macrophages that debride and sterilize the wound and fibroblasts that begin synthesis of ECM; 3) causing deposition of new ECM by simultaneously stimulating the synthesis of new ECM, inhibiting the proteases that degrade matrix and modulating the numbers of integrin receptors to facilitate cell adhesion to the newly assembled matrix; 4) suppressing the proinflammatory effects of interleukin-1 and tumor necrosis factor; 5) regulating the action of platelet derived growth factor and fibroblast growth factor so that cell proliferation and angiogenesis are coordinated with matrix deposition; and 6) terminating the process when repair

is complete and the wound is closed (Border and Noble, *Scientific Amer. Sci. & Med.* 2:68-77 (1995)).

Interactions between RAS and TGFβ occur at both the systemic and molecular level. It has been shown that TGFβ's action in causing ECM deposition in a healing wound is the same action that makes TGFβ a powerful fibrogenic cytokine. (Border and Noble, *New Engl. J. Med.* 331:1286-1292 (1994); and Border and Ruoslahti, *J. Clin. Invest.* 90:107 (1992)). Indeed, it is the failure to terminate the production of TGFβ that distinguishes normal tissue repair from fibrotic disease. RAS and TGFβ co-regulate each other's expression. Thus, both systems may remain active long after an emergency response has been terminated, which can lead to progressive fibrosis. The kidney is particularly susceptible to overexpression of TGFβ. The interrelationship of RAS and TGFβ may explain the susceptibility of the kidney to TGFβ overexpression and why pharmacologic suppression of RAS or inhibition of TGFβ are both therapeutic in fibrotic diseases of the kidney. (Noble and Border, *Sem. Nephrol.*, supra and Border and Noble, *Kidney Int.* 51:1388-1396 (1997)).

Activation of RAS and generation of angiotensin II (AII) are known to play a role in the pathogenesis of hypertension and renal and cardiac fibrosis. TGFβ has been shown to be a powerful fibrogenic cytokine, acting simultaneously to stimulate the synthesis of ECM, inhibit the action of proteases that degrade ECM and increasing the expression of cell surface integrins that interact with matrix components. Through these effects, TGFβ rapidly causes the deposition of excess ECM. AII infusion strongly stimulates the production and activation of TGFβ in the kidney. (Kagami et al., *J. Clin. Invest.* 93:2431-2437 (1994)). Angiotensin II also upregulates TGFβ production and increases activation when added to cultured vascular smooth muscle cells (Gibbons et al, *J. Clin. Invest.* 90:456-461 (1992)) and this increase is independent of pressure (Kagami et al., supra). AII also upregulates TGFβ receptors, even in the presence of exogenously added TGFβ which normally down-regulates its own receptors, leading to enhanced TGFβ signalling and enhanced fibronectin production (Kanai et al., *J. Am. Soc. Nephrol.* 8:518A (1997)). Blockade of AII reduces TGFβ overexpression in kidney and heart, and it is thought that TGFβ mediates renal and cardiac fibrosis associated with activation of RAS (Noble and Border, *Sem. Nephrol.* 17(5):455-466 (1997)), Peters et al., *Kidney International* 54 (1998)).

Blockade of AII using inhibitors of ACE slow the progression of renal fibrotic disease (see, e.g., Anderson et al., *J. Clin. Invest.* 76:612-619 (1985) and Noble and Border, *Sem. Nephrol.* 17(5):455-466 (1997)). What is not clear is whether angiotensin blockade reduces fibrosis solely through controlling glomerular hypertension and thereby glomerular injury, or whether pressure-independent as well as pressure-dependent mechanisms are operating. While ACE inhibitors and AII receptor antagonists have been shown to slow the progress of fibrotic diseases, they do not halt disease and TGFβ levels remain somewhat elevated. (Peters et al., supra).

Thus, RAS and TGFβ can be viewed as powerful effector molecules that interact to preserve systemic and tissue homeostasis. The response to an emergency such as tissue injury is that RAS and TGFβ become activated. Continued activation may result in chronic hypertension and progressive tissue fibrosis leading to organ failure. Because of the interplay between the RAS and TGFβ, and the effects of this interplay on tissue homeostasis, blockade of the RAS may be suboptimal to prevent or treat progressive fibrotic diseases such as diabetic nephropathy.

Components of the renin-angiotensin system act to further stimulate production of TGFβ and plasminogen activator inhibitor leading to rapid ECM accumulation. The protective effect of inhibition of the renin-angiotensin system in experimental and human kidney diseases correlates with the suppression of TGFβ production.(Noble and Border, *Sem. Nephrol.*, supra; and Peters et al., supra).

The renin molecule has been shown to enzymatically cleave angiotensinogen into Angiotensin I. The angiotensin I is then converted by Angiotensin Converting Enzyme ("ACE") to Angiotensin II which acts as an active metabolite and induces TGFβ production. Angiotensin II is an important modulator of systemic blood pressure. It has been thought that if you decrease hypertension by blocking AII's vasoconstrictor effects fibrotic disease is reduced.

In the glomerular endothelium, activation of RAS and TGFβ have been shown to play a role in the pathogenesis of glomerulonephritis and hypertensive injury. Volume (water) depletion and restriction of potassium have been shown to stimulate both production of renin and

TGFβ in the juxtaglomerular apparatus (JGA) of the kidney (Horikoshi et al., *J. Clin. Invest.* 88:2117-2122 (1992) and Ray et al., *Kidney Int.* 44:1006-1013 (1993)). Angiotensin blockade has also been shown to increase the production of renin. TGFβ has been shown to stimulate the release of renin from kidney cortical slices and cultured JG cells (Antonipillai et al., *Am. J. Physiol.* 265:F537-F541 (1993); Ray et al., *Contrib. Nephrol.* 118:238-248 (1996) and Veniant et al., *J. Clin. Invest.* 98:1996-19970 (1996)), suggesting that renin and TGFβ are coregulated. Other interactions between RAS and TGFβ include that AII induces the production of TGFβ in cultured cells and in vivo (Kagami et al., supra) and AII regulates expression of TGFβ receptors (Kanai et al., 1977, supra). It is thus likely that the fibrogenic effects that have been attributed to AII are actually mediated by TGFβ.

Another interplay between RAS and TGFβ is with the production of aldosterone. Aldosterone overproduction has been linked to hypertension and glomerulosclerosis. AII stimulates the production and release of aldosterone from the adrenal gland. In contrast, TGFβ suppresses aldosterone production and blocks the ability of AII to stimulate aldosterone by reducing the number of AII receptors expressed in the adrenal (Gupta et al., *Endocrinol.* 131:631-636 (1992)), and blocks the effects of aldosterone on sodium reabsorption in cultured renal collecting duct cells (Husted et al., *Am. J. Physiol. Renal, Fluid Electrolyte Physiol.* 267:F767-F775 (1994)). Aldosterone may have fibrogenic effects independent of AII, and may upregulate TGFβ expression. The mechanism of aldosterone's pathological effects is unknown but might be due to stimulation of TGFβ production in the kidney (Greene et al., *J. Clin. Invest.* 98:1063-1068 (1996)).

Prorenin or renin may have AII-independent actions to increase fibrotic disease. Prorenin overexpressing rats were found to be normotensive but to develop severe glomerulosclerosis (Veniant et al., *J. Clin. Invest.* 98:1996-1970 (1996)).

Human recombinant renin added to human mesangial cells induces marked upregulation of production of plasminogen activator inhibitors (e.g. PAI-1 and PAI-2) which block the generation of plasmin, a fibrinolytic enzyme important in the dissolution of clots after wounding generated from plasminogen by two enzymes called plasminogen activators, urokinase (u-PA)

and tissue plasminogen activator (t-PA). PAI-1 and 2 regulate U-PA and t-PA in turn. Plasmin appears to be a key mediator of extracellular matrix degradation, carrying out at least three functions important to extracellular matrix degradation. Plasmin directly degrades proteoglycan components of extracellular matrix, proteolytically activates metalloproteinases (MMPs) that, in turn, degrade collagens and other matrix proteins, and enzymatically inactivates tissue inhibitors of MMPs (TIMPs), releasing MMPs from inhibition of TIMPs, allowing them to proteolytically digest matrix proteins. (Baricos et al., *Kidney Int'l.* 47:1039-1047 (1995); Baricos et al., *J. Amer. Soc. Nephrol.* 10:790-795 (1999)). The net generation of active plasmin from the inactive precursor plasminogen results from a balance of the plasminogen activators and PAI-1 and 2, and other factors. PAI-1 binds to vitronectin. (Lawrence et al., *J. Biol. Chem.* 272:7676-7680 (1997)). Mutant PAI-1 molecules have been developed that have enhanced properties for PAI-1 binding to vitronectin molecules, but do not inhibit either t-PA or u-PA activity, resulting in an increase in the amount of the active form of plasmin. (See, WO 97/39028, Lawrence et al.). PAI-1 is increased in response to added TGFβ (Tomooka et al., *Kidney Int.* 42:1462-1469 (1992)).

It has been suggested that TGFβ enhances release of renin from storage granules in the juxtaglomerular apparatus of the kidney (Antonipillai et al., *Am. J. Physiol.* 265:F537-F541 (1993) and Ray et al., *Contrib. Nephrol.* 118:238-248 (1996)).

Thus, the interactions of RAS and TGFβ production form a complex system which impacts fibrotic ECM accumulation and the incidence of fibrotic disease. Various RAS components such as aldosterone, prorenin and renin may be connected with TGFβ production and fibrotic ECM accumulation. Any successful therapeutic regime must take into account these complex relationships to optimize inhibition of TGFβ to prevent and/or reduce ECM accumulation.

The multiple pathways resulting in TGFβ overexpression and fibrosis proposed from in vitro studies are depicted in Figure 1. (See, Kagami et al., *J. Clin. Invest.* 93:2431-2437 (1994); Gibbons et al., *J. Clin. Invest.* 90:456-461 (1992); Abboud, *Kidney Int.* 41:581-583 (1992); Ruiz-Ortega et al., *J. Am. Soc. Nephrol.* 5:683 (1994) abstract; Kim et al., *J. Biol. Chem.* 267:13702-

13707 (1992); Ohno et al., *J. Clin. Invest.* 95:1363-1369 (1995); Riser et al, *J. Clin. Invest.* 90:1932-1943 (1992); Riser et al., *J. Am. Soc. Nephrol.* 4:663 (1993); Ziyadeh et al., *J. Clin. Invest.* 93:536-542 (1994); Rocco et al., *Kidney Int.* 41:107-114 (1992); Flaumenhaft et al., *Advan. Pharmacol.* 24:51-76 (1993); Lopez-Armanda et al., *J. Am. Soc. Nepbrol.* 5:812 (1994) abstract; Sahai et al., *J. Am. Soc. Nephrol.* 6:910 (1995); Remuzzi et al., *Kidney Int.* 1:2-15 (1997); and Remuzzi et al., *J. Am. Soc. Nephrol.* 9:1321-1332 (1998)). This diagram shows that a large number of factors implicated in kidney injury are believed to increase the production of TGFβ.

In fibrotic diseases overproduction of TGFβ results in excess accumulation of extracellular matrix which leads to tissue fibrosis and eventually organ failure. Accumulation of mesangial matrix is a histological indication of progressive glomerular diseases that lead to glomerulosclerosis and end-stage kidney disease (Klahr et al., *N. Engl. J. Med.* 318:1657-1666 (1988); Kashgarian and Sterzel, *Kidney Int.* 41:524-529 (1992)). Rats injected with antithymocyte serum are an accepted model of human glomerulonephritis and this model has demonstrated that overproduction of glomerular TGFβ can underlie the development of glomerulosclerosis (Okuda et al., *J. Clin. Invest.* 86:453-462 (1990); Border et al., *Nature (Lond.)* 346:371-374 (1990); Kagami et al., *Lab. Invest.* 69:68-76 (1993); and Isaka et al., *J. Clin. Invest.* 92:2597-2602 (1993)). Using cultured rat mesangial cells where the effects of Angiotensin II on glomerular pressure are not a factor, Angiotensin II has been shown to induce TGFβ production and secretion by mesangial cells, and this in turn has been shown to stimulate extracellular matrix production and deposition (Kagami et al., *J. Clin. Invest.* 93:2431-2437 (1994)). Increases in PAI-1 levels result in decreased degradation of extracellular matrix (Baricos et al., *Kidney Int.* 47:1039-1047 (1995)). Increases in TGFβ result in increased PAI-1 levels (Tomooka et al., *Kidney Int.* 42:1462-1469 (1992)). It has been demonstrated that decreasing TGFβ overexpression in a rat model of glomerulonephritis by in vivo injection of neutralizing antibodies to TGFβ, reduces TGFβ overexpression (Border et al., *Nature* 346:371-374 (1990)), and reduces PAI-1 deposition into the pathological matrix (Tomooka et al., *Kidney Int.* 42:1462-1469 (1992)). Therefore, decreases in TGFβ levels should result in decreased PAI-1 levels and increased degradation of extracellular matrix to ameliorate organ impairment and fibrotic disease. However, patients present with fibrotic disease that is well advanced in terms of build-up of

extra-cellular matrix (ECM). This is because abnormal organ function is undetectable until ECM accumulation is very advanced. For example, in the kidney, standard diagnostic tests do not provide an abnormal reading until about fifty percent of organ function has been lost.

The treatment of conditions associated with excess accumulation of ECM has also focused on decreasing stimuli to disease such as to lower blood pressure or, in the case of diabetic nephropathy to reduce plasma glucose levels. For example, current therapies for treating fibrotic disease in the kidney are limited to AII blockade using ACE inhibitors such as Enalapril or AII receptor antagonists such as Losartan. In addition, patients are encouraged to follow low protein diets since this regimen has some therapeutic value (Rosenberg et al., *J. Clin. Invest.* 85:1144-1149 (1992)). These therapies, at best, prolong organ function by only 1-2 years. This may be because of the multiple pathways that result in TGFβ overexpression or enhanced activity. Moreover, it is likely that current therapeutic strategies to reduce TGFβ overproduction may lead to upregulation of other pathways resulting in continued TGFβ overproduction. For example, when the action of AII is blocked, renin is upregulated which itself increases TGFβ production (see co-pending U.S. patent application, U.S. Serial No. 09/005,255, incorporated in its entirety herein). More recently, treatments aimed to halt the overproduction of TGFβ have been proposed (Border and Noble, *Kidney Internatl.* 54 (1998); and Peters et al., *Kidney Internatl.* 54 (1998)).

Therefore, the most promising therapeutic methods will need to increase ECM degradation to restore organ function as well as decrease TGFβ overproduction and/or activity. Enhanced degradation of excess accumulated ECM can be used to optimize overall reduction in levels of accumulated ECM to restore function to tissues and organs. Proteases that are able to degrade ECM are known. For example, the serine protease plasmin degrades ECM proteins and activates pro-metalloproteinases, in addition to degrading fibrin (Baricos et al., supra). One goal of therapeutic intervention to increase ECM degradation for treating fibrosis could be increasing plasmin in the region of excess ECM deposition.

There is a need for improved therapies to normalize TGFβ production, that take into account the multiple pathways that stimulate TGFβ production, to prevent or reduce excess

accumulation of ECM, to restore function to tissues and organs in which excess ECM has accumulated and/or to reduce scar formation at a wound site.

SUMMARY OF THE INVENTION

Accordingly, the present invention provides methods for preventing or reducing the excess accumulation of extracellular matrix (ECM) associated with fibrotic conditions by inhibiting TGFβ, using a combination of agents that inhibit TGFβ, or by using a combination of agents to inhibit TGFβ and agents that cause the enhanced degradation of excess accumulated ECM.

The methods of the invention contemplate the use of agents that directly or indirectly inhibit TGFβ including direct inhibitors of TGFβ activity such as anti-TGFβ antibodies, proteoglycans such as decorin and ligands for TGFβ receptors, and/or indirect TGFβ inhibitors including aldosterone, inhibitors of aldosterone, inhibitors of angiotensin II, renin inhibitors, ACE inhibitors and AII receptor antagonists which act to decrease TGFβ production.

The methods of the invention also contemplate the use of agents that result in the enhanced degradation of excess accumulated matrix including proteases such as serine proteases including plasmin, metalloproteases, or protease combinations, and agents such as tPA, and PAI-1 mutants that increase the production and/or the activity of proteases such as plasmin.

The agents for use in the methods of the invention may be administered as inhibitory compounds in pharmaceutical formulations or as nucleic acid encoding the inhibitors delivered to suitable host cells. The nucleic acid may be directly introduced into a cell in vivo, for example into muscle tissue, or may be first introduced into a cell ex vivo to obtain a cell expressing the inhibitory agent or agents, and the cell then transplanted or grafted into a subject to inhibit or reduce excess accumulation of extracellular matrix.

The invention includes compositions for preventing or reducing the excess accumulation of ECM containing a combination of agents for inhibiting TGFβ or a combination of agents for inhibiting TGFβ and for degrading ECM.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram depicting various pathways resulting in increased TGFβ production.

Figure 2 is a bar graph showing increases in TGFβ production by cultured human mesangial cells in response to renin, as described in Example I, infra.

Figure 3 is a bar graph showing the effect of blocking agents on TGFβ-production by human mesangial cells in response to renin, as described in Example II, infra.

Figure 4A and B are bar graphs showing dose dependent increases in TGFβ (Figure 4A) and Fn production (Figure 4B) with increases in HrRenin as described in Example IV, infra.

Figure 5A and B are bar graphs showing time courses of TGFβ (Figure 5A) and Fn production (Figure 5B) as described in Example IV, infra.

Figure 6A-C are bar graphs showing renin-induced increases in TGFβ, PAI-1 and Fn mRNAs over time as described in Example IV, infra.

Figure 7 is a bar graph showing the results of inhibitors that block renin's action to increase Angiotensin II, on the renin-induced increase in TGFβ production in adult human mesangial cells as described in Example IV, infra.

Figure 8A and B are photographs depicting the effects of tPA treatment on ECM accumulation in glomeruli as described in Example V, infra.

Figure 9A-D are bar graphs depicting the effects of tPA treatment on amounts of ECM

constituents (9A: FN EDA+; 9B:Laminin; 9C:Collagen I and 9D:Collagen IV) as determined by staining as described in Example V, infra.

Figure 10 is a bar graph showing the effects of tPA on glomerular mRNA expression at day 6 as described in Example V, infra.

Figure 11A and B are bar graphs showing the effects of tPA treatment on glomerular plasmin activity as described in Example V, infra.

Figure 12 is a bar graph demonstrating that injection of PAI-1 mutant results in increases in plasmin generation of nephritic glomeruli, as described in Example VII, infra.

Figure 13 is a bar graph demonstrating decreased accumulation of Collagen type I after administration of PAI-1 mutant, as described in Example VII, infra.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the discovery that a combination of strategies may be warranted to prevent or treat conditions associated with the excess accumulation of extracellular matrix in tissues or organs, including fibrotic diseases and scarring resulting from TGFβ overproduction and/or activity. As previously reported, TGFβ overproduction may result from multiple pathways and require that more than one pathway be inhibited to achieve any clinically significant reduction in excess accumulation of extracellular matrix and amelioration of disease. For example, as disclosed in co-pending U.S. patent application, U.S. Serial No. 09/005,255, incorporated in its entirety herein, renin stimulates TGFβ production in cells capable of producing TGFβ, in an angiotensin-II and blood pressure-independent manner.

Optimal therapy of disorders associated with excess accumulation of ECM which causes organ impairment and ultimately failure, must take into account the multiple pathways of TGFβ production to effectively combat overproduction of TGFβ. Without such multifactorial strategy,

inhibition of one pathway of TGFβ production may be insufficient to block excess accumulation of extracellular matrix and can even result in an increase in the levels of TGFβ production by stimulation of one of the alternative pathways for its production.

While it is now known that multiple stimuli result in TGFβ overexpression and resulting excess accumulation of ECM, therapeutic strategies directly inhibiting TGFβ, such as the use of anti-TGFβ antibodies or TGFβ receptor antagonists, are being explored. However, because TGFβ has many beneficial actions such as immunosuppressive and immunomodulatory effects, as well as inhibition of epithelial cell growth which retards carcinogenesis (Markowitz, *Science* 268:1336-1338 (1995) and suppression of atherogenesis (Grainger et al., *Nature Med.*1:74-79 (1995), these therapies may have unacceptable side-effects if administered at doses high enough to successfully stem fibrotic conditions. This has been shown in the TGFβ1 null (knockout) mice which die of overwhelming inflammation at about 6 weeks of age (Letterio et al., *Science* 264:1936-1938 (1994); Kulkami et al, *Proc. Natl. Acad. Sci. USA* 90:770-774 (1993) and Shull et al., *Nature* 359:693-699 (1992)), indicating that TGFβ1 has significant beneficial roles in immune function. Multiple agents, inhibiting TGFβ directly, and/or inhibiting the disease-specific stimuli underlying TGFβ overexpression and/or activity, for example high glucose resulting from diabetes, may be required to adequately reduce TGFβ-associated excess accumulation of ECM, without causing harmful side-effects. Accordingly, it is a goal of the methods of the present invention to accomplish normalization of TGF production without harmful side effects and to prevent or reduce excess accumulation of ECM and ensuing fibrotic conditions.

In addition, degradation of accumulated ECM may be needed to restore tissue or organ function that has been compromised by the presence of the excess accumulated ECM. Prevention or degradation of excess accumulated ECM can also prevent or reduce scar formation at the site of a wound.

The methods of the invention include using multiple agents to reduce the overproduction and/or activity of TGFβ and/or to block alternative pathways of TGFβ production to prevent or reduce excess accumulation of ECM. The methods of the invention further include the use of

a combination of agents to reduce TGFβ overproduction and/or activity in combination with agents to enhance the degradation of excess, accumulated ECM. The methods are useful to prevent or reduce excess accumulation of extracellular matrix to ameliorate fibrotic conditions, and to restore or maintain normal tissue or organ function or skin appearance.

As used herein "excess accumulation of extracellular matrix" means the deposition of extracellular matrix components including, collagen, laminin, fibronectin and proteoglycans in tissue to an extent that results in impairment of tissue or organ function and ultimately, organ failure as a result of fibrotic disease. In addition, "excess accumulation of extracellular matrix" means the deposition of extracellular matrix components in the process commonly referred to as "scarring" or "scar formation," e.g. at a wound site. "Reducing the excess accumulation of extracellular matrix" means preventing excess accumulation of extracellular matrix, e.g. in tissue, organs or at a wound site, preventing further deposition of extracellular matrix and/or decreasing the amount of excess accumulated matrix already present, to maintain or restore tissue or organ function or appearance.

A variety of conditions are characterized by excess accumulation of extracellular matrix (collagen, fibronectin and other matrix components). Such conditions include, for example, but are not limited to, glomerulonephritis, adult or acute respiratory distress syndrome (ARDS), diabetes-associated pathologies such as diabetic kidney disease, fibrotic diseases of the liver, lung and post infarction cardiac fibrosis. Also included are fibrocystic diseases such as fibrosclerosis and fibrotic cancers such as cancers of the breast, uterus, pancreas or colon, and including fibroids, fibroma, fibroadenomas and fibrosarcomas.

There are also a number of medical conditions associated with an excess accumulation of extracellular matrix involving increased collagen, fibronectin and other matrix components. Such conditions include, for example, but are not limited to, post myocardial infarction, left ventricular hypertrophy, pulmonary fibrosis, liver cirrhosis, veno-occlusive disease, post-spinal cord injury, post-retinal and glaucoma surgery, post-angioplasty restenosis and renal interstitial fibrosis, arteriovenous graft failure, excessive scarring such as keloid scars and scars resulting from injury, burns or surgery.

As discussed, supra, it is known that TGFβ is indicated in the causation of fibrotic conditions. During normal tissue repair, TGFβ production is increased to stimulate the process of repair. When repair is complete, TGFβ production is reduced. If not reduced following normal tissue repair, the increased TGFβ overproduction can result in the development of excess extracellular matrix accumulation and fibrotic conditions. Thus, repeated tissue injury or a defect in TGFβ regulation leading to sustained TGF production results in excess accumulation of extracellular matrix.

As used herein "inhibition of TGFβ" includes inhibition of TGFβ activity, for example in causing excess deposition of ECM, as well as inhibition of TGFβ production resulting in overproduction and excess accumulation of ECM, regardless of the mechanism of TGFβ activity or overproduction. This inhibition can be caused directly, e.g. by binding to TGFβ or its receptors, for example by anti-TGFβ antibodies or TGFβ receptor antagonists, or can be caused indirectly, for example by inhibiting a pathway that results in TGFβ production, such as the renin pathway. Inhibition causes a reduction in the ECM producing activity of TGFβ regardless of the exact mechanism of inhibition.

As used herein a "TGFβ inhibitory agent" is an agent that directly or indirectly inhibits TGFβ binding to its receptors, such as a TGFβ-specific inhibitory agent, or an agent that blocks an alternative pathway of TGFβ production. The agent causes a reduction in the ECM producing activity of TGFβ regardless of the mechanism of its action. The agent can be nucleic acid encoding the TGFβ inhibitory agent such as a cDNA, genomic DNA, or an RNA or DNA encoding TGFβ inhibitory activity such as a TGFβ antisense RNA or DNA.

As used herein, a "TGFβ-specific inhibitory agent" means an agent containing TGFβ inhibiting activity, including agents that bind directly to TGFβ such as anti-TGFβ antibodies, or are a ligand for TGFβ which prevents it from binding to its receptors. A TGFβ-specific inhibiting agent also includes a nucleic acid encoding a particular TGFβ-specific inhibitory agent such as a cDNA, genomic DNA or an RNA or DNA encoding TGFβ-specific inhibitory activity such as a TGFβ antisense RNA or DNA.

Agents that bind directly to TGFβ are known and include anti-TGFβ antibodies such as anti-TGFβ1 antibodies (Genzyme, Cambridge, MA) and antibodies which bind both TGFβ1 and TGFβ2 (Dasch et al., U.S. Patent No. 5,571,714), proteoglycans such as decorin, biglycan and fibromodulin, and the nucleic acids encoding such agents.

Antibodies to inhibit TGFβ, renin or other molecules, for use in the present invention, can be prepared according to methods well established in the art, for example by immunization of suitable host animals with the selected antigen, e.g. TGFβ. For descriptions of techniques for obtaining monoclonal antibodies see, e.g. the hybridoma technique of Kohler and Milstein (*Nature* 256:495-497 (1975)), the human B-cell hybridoma technique (Kosbor et al., *Immunol. Today* 4:72 (1983); Cole et al., *Proc. Nat'l. Acad. Sci. USA*, 80:2026-2030 (1983)) and the EBV-hybridoma technique (Cole et al., *Monoclonal antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77096 (1985)). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the monoclonal antibody may be cultivated *in vitro* or *in vivo*. Suitable host animals include, but are not limited to, rabbits, mice, rats, and goats. Various adjuvants may be used to increase the immunological response to the host animal, depending on the host species, including, but not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpit, hemocyanin, dinitrophenol and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and Cornebacterium parvum. Antibodies as used herein includes non-human, chimeric (different species), humanized (see Borrebaeck, Antibody Engineering: A Practical Guide, W.H. Freeman and Co., New York, 1991), human and single-chain antibodies, as well as antibody fragments including but not limited to the $F(ab')_2$ fragments that can be produced by pepsin digestion of antibody molecules and Fab fragments that can be generated by reducing disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries may be constructed (*Science* 246:1275-1281 (1989)) to permit the rapid and easy identification of monoclonal Fab fragments having the desired specificity.

An indirect TGFβ inhibitor would inhibit the synthesis or secretion of TGFβ or sequester it away from its target cells. Such inhibitors include, but are not limited to, inhibitors of

Angiotensin Converting Enzyme ("ACE"), antagonists of the AII receptor such as Losartan™ and Cozar™ (Merck), and aldosterone inhibitors such as Spironolactone™ (Sigma Chemical Co., St. Louis, Mo, Product # S 3378) that would otherwise result in increased TGFβ production.

Also included within the scope of TGFβ inhibitors of the invention are nucleic acids that include antisense oligonucleotides that block the expression of specific genes within cells by binding a complementary messenger RNA (mRNA) and preventing its translation (See review by Wagner, *Nature* 372:332-335 (1994); and Crooke and Lebleu, *Antisense Research and Applications*, CRC Press, Boca Raton (1993)). Gene inhibition may be measured by determining the degradation of the target RNA. Antisense DNA and RNA can be prepared by methods known in the art for synthesis of RNA including chemical synthesis such as solid phase phosphoramidite chemical synthesis or in vitro and in vivo transcription of DNA sequences encoding the antisense RNA molecule. The DNA sequences may be incorporated into vectors with RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly can be introduced into cell lines. The potency of antisense oligonucleotides for inhibiting TGFβ may be enhanced using various methods including 1) addition of polylysine (Leonetti et al., *Bioconj. Biochem.* 1:149-153 (1990)); 2) encapsulation into antibody targeted liposomes (Leonetti et al., *Proc. Natl. Acad. Sci. USA* 87:2448-2451 (1990) and Zelphati et al., *Antisense Research and Development* 3:323-338 (1993)); 3) nanoparticles (Rajaonarivony et al., *J. Pharmaceutical Sciences* 82:912-917 (1993) and Haensler and Szoka, *Bioconj. Chem.* 4:372-379 (1993)), 4) the use of cationic acid liposomes (Felgner et al., *Proc. Natl. Acad. Sci. USA* 84:7413-7417 (1987); Capaccioli et al., *Biochem. Biophys. Res. Commun.* 197:818-825 (1993); Boutorine and Kostina, *Biochimie* 75:35-41 (1993); Zhu et al., *Science* 261:209-211 (1993); Bennett et al., *Molec. Pharmac.* 41:1023-1033 (1992) and Wagner, *Science* 280:1510-1513 (1993)); and 5) Sendai virus derived liposomes (Compagnon et al., *Exper. Cell Res.* 200:333-338 (1992) and Morishita et al., *Proc. Natl. Acad. Sci. USA* 90:8474-8478 (1993)), to deliver the oligonucleotides into cells. Recent techniques for enhancing delivery include the conjugation of the antisense oligonucleotides to a fusogenic peptide, e.g. derived from an influenza hemagglutinin envelop protein (Bongartz et al., *Nucleic Acids Res.* 22(22):4681-4688 (1994)).

Additional suitable TGFβ inhibitory agents can be readily obtained using methods known in the art to screen candidate agent molecules for binding to TGFβ, such as assays for detecting the ability of a candidate agent to block binding of radiolabeled human TGFβ to cells such as human mesangial cells. Alternatively, candidate compounds may be tested for the ability to inhibit TGFβ production by mesangial cells using an enzyme-linked immunosorbent assay (ELISA), for example using the R & D Systems (Minneapolis, MN) TGFβ ELISA assay kit (Cat. No. DB 100) (for methods see, e.g. Uotila et al., *J. Immunol. Methods* 42:11 (1981)).

Suitable TGFβ-specific inhibitory agents can also be developed by known drug design methods, e.g. using structural analysis of the TGFβ molecule employing methods established in the art, for example, using X-ray crystallography to analyze the structure of the complex formed by TGFβ and one of its known inhibitors (see, e.g. Sielecki et al., supra; Rahuel et al., supra, Badasso et al., supra and Dhanaraj et al., supra.), and/or by modifying known TGFβ antagonists i.e. "lead compounds," to obtain more potent inhibitors and compounds for different modes of administration (i.e. oral vs. intravenous) (see, e.g. Wexler et al., *Amer. J. Hyper.* 5:209S-220S (1992)-development of AII receptor antagonists from Losartan™). For such procedures large quantities of TGFβ can be generated using recombinant technology or purchased commercially (R & D Systems).

In addition to TGFβ inhibitory agents, agents that result in the degradation of ECM are contemplated for use in the invention. Such agents include serine proteases such as plasmin and metalloproteinases, and protease combinations such as Wobenzym (Mucos Pharma, Geretsried, Germany) . In addition, the present inventors have discovered that agents such as tPA can be used to increase the amount of active proteases in vivo to increase degradation of ECM accumulated in organs and tissues. Tissue plasmin activator (tPA, Activase, Genentech, S. San Francisco, CA) has been shown to dissolve clots associated with myocardial infarction and stroke. The present inventors theorized that tPA might be helpful in increasing plasmin to reduce accumulated ECM. Shown herein is the use of recombinant tPA (rtPA) to increase the generation of plasmin in vivo to degrade ECM (Example V, infra).

In addition, new proteases or agonists of protease production and/or activity may be discovered or developed using rational drug design and used to degrade ECM according to the methods of the present invention.

The present inventors have also discovered that PAI mutants, such as the PAI-1 mutants disclosed in WO 97/39028 by Lawrence et al., incorporated by reference in its entirety herein, may be used to increase the amount of active plasmin to enhance degradation of ECM accumulated in organs and tissues. These PAI-1 mutants fail to inhibit plasminogen activators, yet retain significant vitronectin binding affinity. Additional PAI-1 mutants for use in the methods of the invention may be obtained and tested for the ability to bind vitronectin while failing to inhibit plasminogen activators (Lawrence et al., *J. Biol. Chem.* 272:7676-7680 (1997)). PAI-1 binding to vitronectin may be determined either functionally (Lawrence et al., *J. Biol. Chem.* 265:20293-20301 (1990)) or in a vitronectin specific ELISA (Lawrence et al., *J. Biol. Chem.* 269:15223-15228 (1994)). The ability of PAI-1 to inhibit plasminogen activators may be evaluated using chromogenic assays as described by Sherman et al., *J. Biol. Chem.* 270:9301-9306 (1995)).

In the methods of the invention, the TGFβ inhibitory agents are administered concurrently or sequentially. For example, an anti-TGFβ antibody is administered with an anti-renin agent. The inhibitory agents will localize at sites of TGFβ overproduction, e.g. organs such as the kidneys. The inhibitory agents may be labelled, using using known radiolabelling methods to detect their localization in a subject after administration. The agents may also be conjugated to targeting molecules such as antibodies to ECM components to improve localization of the agents after administration to the sites of TGFβ overproduction and/or excess accumulation of ECM in a subject.

In another embodiment of the methods of the invention, TGFβ inhibitory agents are administered concurrently or sequentially with at least one agent that degrades accumulated ECM, for example, a serine protease such as plasmin. Alternatively, an agent that induces protease production, such as tPA, is administered to increase protease production at the site(s) of accumulated ECM. tPA binds fibrin (Rondeau et al., *Clinical Nephrol.* 33:55-60 (1990)) and

thus will localize in fibrotic areas where the increased protease production is desired.

In one embodiment of the invention, at least one TGFβ-inhibitory agent is administered to a subject having existing excess accumulation of ECM in tissues or organs, or at high risk for such accumulation to reduce or prevent excess accumulation of ECM. For example, individuals at risk for developing fibrotic conditions, such as a person having or at high risk for diabetes, high blood pressure, autoimmune disease (e.g. lupus) and inflammatory diseases, can be scanned using known medical procedures including tissue biopsies of kidney, lung or liver, to determine whether ECM has accumulated in these organs. If the agent is TGFβ-specific, it binds to circulating TGFβ or tissue TGFβ. If the agent indirectly inhibits TGFβ, for example an anti-renin agent, it reduces the amount of TGFβ produced. As a result of the administration of agents that directly or indirectly inhibits TGFβ, ECM that has accumulated at the time of diagnosis or treatment, as well as further accumulation of ECM is reduced. Moreover, in high risk individuals the methods of the invention for inhibiting TGFβ overproduction with multiple agents can result in prevention of excess accumulation of ECM and the development of fibrotic conditions.

In another embodiment of the methods of the invention, at least one TGFβ inhibitory agent is administered to a subject having an existing excess accumuation of ECM in tissues or organs together with at least one agent to degrade accumulated ECM. The ECM degradation is accomplished using a protease, or an agent that enhances production or the activity of ECM degrading agents such as proteases. As a result of the administration of these agents, excess matrix accumulated at the time of diagnosis or treatment, as well as further excess accumulation of ECM is reduced.

In addition to the use of molecules such as antibodies and purified compounds such as decorin, nucleic acid encoding the TGFβ inhibitory agents and nucleic acid encoding the agent to directly or indirectly degrade accumulated ECM, are administered to the subject to permit the agents to be expressed and secreted, for inhibiting TGFβ and degrading accumulated ECM. The nucleic acid may be introduced into cells in the subject, for example using a suitable delivery vehicle such as an expression vector or encapsulation unit such as a liposome, or may be introduced directly through the skin, for example in a DNA vaccine.

Alternatively, the nucleic acids encoding the agents are introduced into a cell ex vivo and the cells expressing the nucleic acids are introduced into a subject, e.g. by implantation procedures, to deliver the agents in vivo. Multiple agents can be introduced into a delivery vehicle or in separate vehicles.

Gene Therapy Methods

Methods for obtaining nucleic acids encoding TGFβ inhibitory agents and ECM degrading agents are known in the art. Following is a general description of methods of using the nucleic acids in gene therapy to reduce excess accumulation of ECM.

In one embodiment of the invention, gene therapy is contemplated using nucleic acids encoding the TGFβ inhibitory agents and/or the ECM degradation agent, introduced into cells in a subject to suppress TGFβ overproduction and to degrade accumulated ECM. Gene transfer into cells of these nucleic acids is contemplated in the methods of the invention.

Nucleic Acids

Large amounts of the nucleic acid sequences encoding the TGFβ-inhibiting agents and/or the ECM degradation agents may be obtained using well-established procedures for molecular cloning and replication of the vector or plasmid carrying the sequences in a suitable host cell. DNA sequences encoding a specific agent can be assembled from cDNA fragments and oligonucleotide linkers, or from a series of oligonucleotides to provide a synthetic inhibitor agent gene and/or ECM degradation gene which can be expressed. Such sequences are preferably provided in an open reading frame uninterrupted by internal non-translated sequences or introns, which are typically present in eukaryotic genes. Genomic DNA containing the relevant sequences can also be used. Sequences of non-translated DNA may be present 5' to 3' from the open reading frame, where such sequences do not interfere with manipulation or expression of the coding regions. Either complete gene sequences or partial sequences encoding the desired agents are employed.

The nucleic acid sequences encoding the agents can also be produced in part or in total by chemical synthesis, e.g. by the phosphoramidite method described by Beaucage and Carruthers, *Tetra Letts.* 22:1859-1862 (1981) or the triester method (Matteucci et al., *J. Am. Chem. Soc.* 103:3185 (1981) and may be performed on commercial automated oligonucleotide synthesizers. A double-stranded fragment may be obtained from the single-stranded product of chemical synthesis either by synthesizing the complementary strand and annealing the strand together under appropriate conditions, or by synthesizing the complementary strand using DNA polymerase with an appropriate primer sequence.

Gene Transfer

For gene transfer, the key steps are 1) to select the mode of delivery, e.g. a proper vector for delivery of the inhibitor genes to the subject, 2) administer the nucleic acid to the subject; and 3) achieve appropriate expression of the transferred gene for satisfactory durations. Methods for gene transfer are known in the art. The methods described below are merely for purposes of illustration and are typical of those that can be used to practice the invention. However, other procedures may also be employed, as is understood in the art. Most of the techniques to construct delivery vehicles such as vectors and the like are widely practiced in the art, and most practitioners are familiar with the standard resource materials which describe specific conditions, reagents and procedures. The following paragraphs may serve as a guideline.

Techniques for nucleic acid manipulation are well known. (See, e.g. *Annual Rev. of Biochem.* 61:131-156 (1992)). Reagents useful in applying such techniques, such as restriction enzymes and the like, are widely known in the art and commerically available from a number of vendors.

The natural or synthetic nucleic acid coding for the inhibitors for expression in a subject may be incorporated into vectors capable of introduction into and replication in the subject. In general, nucleic acid encoding the selected inhibitor molecules and/or ECM degradation molecules are inserted using standard recombinant techniques into a vector containing appropriate transcription and translation control sequences, including initiation sequences

operably linked to the gene sequence to result in expression of the recombinant genes in the recipient host cells. "Operably linked" means that the components are in a physical and functional relationship permitting them to function in their intended manner.

For example, a promoter is operably linked to a coding sequence if the promoter effects its transcription or expression.

Sequences encoding selected inhibitor and/or degradation genes will include at least a portion of the coding sequence sufficient to provide the TGFβ inhibitory or ECM degradation activity in the expressed molecule. For example, in the case of a renin inhibitor, a portion of the coding sequence that enables the inhibitor to bind to renin can be used. Methods for determining such portions or "domains" including binding domains of molecules, are known in the art (See, e.g., Linsley et al., *Proc. Natl. Acad. Sci. USA* 87:5031-5035 (1990)). It is possible that it may be necessary to block both the renin enzymatic site and the renin-cell binding domain in order to effectively prevent the stimulus to TGFβ overproduction by renin. In such case, renin antisense molecules can be prepared using standard methods to accomplish complete blockade.

The selected nucleic acid sequences are inserted into a single vector or separate vectors. More than one gene encoding a selected agent, or portion thereof containing the desired activity, may be inserted into a single vector or into separate vectors for introduction into the host cells. Alternatively, these sequences can be administered as naked nucleic acid sequences or as part of a complex with other molecules, e.g. liposomes.

A variety of expression vectors and gene transfer methods useful for obtaining expression of selected molecule in recipient cells are well known in the art, and can be constructed using standard ligation and restriction techniques (see, for example, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1989; Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York (1982), Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (W.H. Freeman and Co., New York, NY 1990) and Wu, *Methods in Enzymol.* (Academic Press, New York, NY 1993), each of which is incorporated by reference herein). The choice of vector or method depends on several factors

such as the particular molecule to be expressed.

Suitable vectors may be plasmid or viral vectors (Kaufman, in *Gene Expression Technology,* Goeddel (Ed.) (1991)) including baculoviruses, adenoviruses, poxviruses (Moss, *Current Opin. Biotech.* 3:518-522 (1993)), retrotransposon vectors (Cook et al., *Bio/Technology* 9:748-751 (1991) and Chakraborty et al., *FASEB J.* 7:971-977 (1993)) adeno-associated viruses (AAV) (Yei et al., *Gene Therapy* 1:192-200 (1994) and Smith et al., *Nat. Genet.* 5:397-402 (1993)), herpes virus and retrovirus vectors (Price et al., *Proc. Natl. Acad. Sci. USA* 84:156-160 (1987); Naviaux and Verma, *Current Opinion in Biotechnol.* 3:540-547 (1992); Hodgson and Chakraborty, *Curr. Opin. Thera. Patients* 3:223-235 (1993)) such as the MMLV based replication incompetent vector pMV-7 (Kirschmeier et al., *DNA* 7:219-225 (1988)), as well as human and yeast artificial chromosomes (HACs and YACs) (Huxley, *Gene Therapy* 1:7-12 (1994) and Huxley et al., *Bio/Technology* 12:586-590 (1994)). Plasmid expression vectors include plasmids including pBR322, pUC or Bluescript™ (Stratagene, San Diego, CA).

Vectors containing the nucleic acid encoding the selected agents are preferably recombinant expression vectors in which high levels of gene expression may occur, and which contain appropriate regulatory sequences for transcription and translation of the inserted nucleic acid sequence. Regulatory sequences refer to those sequences normally associated (e.g. within 50 kb) of the coding region of a locus which affect the expression of the gene (including transcription, translation, splicing, stability or the like, of the messenger RNA). A transcriptional regulatory region encompasses all the elements necessary for transcription, including the promoter sequence, enhancer sequence and transcription factor binding sites. Regulatory sequences also include, inter alia, splice sites and polyadenylation sites. An internal ribosome entry site (IRES) sequence may be placed between recombinant coding sequences to permit expression of more than one coding sequence with a single promoter.

Transcriptional control regions include: the SV40 early promoter region, the cytomegalovirus (CMV) promoter (human CMV IE94 promoter region (Boshart et al., *Cell* 41:521-530 (1985)); the promoter contained in the 3' long terminal repeat of Rous Sarcoma Virus or other retroviruses; the herpes thymidine kinase promoter; the regulatory sequences of the

methallothionein gene; regions from the human IL-2 gene (Fujita et al., *Cell* 46:401-407 (1986)); regions from the human IFN gene (Ciccarone et al., *J. Immunol.* 144:725-730 (1990); regions from the human IFN gene (Shoemaker et al., *Proc. Natl. Acad. Sci. USA* 87:9650-9654 (1990); regions from the human IL-4 gene (Arai et al., *J. Immunol.* 142:274-282 (1989)); regions from the human lymphotoxin gene (Nedwin et al., *Nucl. Acids. Res.* 13:6361-6373 (1985)); regions from the human granulocyte-macrophage CSF gene (GM-CSF) (Miyatake et al., *EMBO J.* 4:2561-2568 (1985)) and others. When viral vectors are used, recombinant coding sequences may be positioned in the vector so that their expression is regulated by regulatory sequences such as promoters naturally residing in the viral vector.

Operational elements for obtaining expression may include leader sequences, termination codons and other sequences needed or preferred for the appropriate transcription and translation of the inserted nucleic acid sequences. Secretion signals may also be included whether from the native inhibitor or from other secreted polypeptides, which permit the molecule to enter cell membranes and attain a functional conformation. It will be understood by one skilled in the art that the correction type and combination of expression control elements depends on the recipient host cells chosen to express the molecules ex vivo. The expression vector should contain additional elements needed for the transfer and subsequent replication of the expression vector containing the inserted nucleic acid sequences in the host cells. Examples of such elements include, but are not limited to, origins of replication and selectable markers. Additionally, elements such as enhancer sequences, for example CMV enhancer sequences, may be used to increase the level of therapeutic gene expression (Armelor. *Proc. Natl. Acad. Sci. USA* 70:2702 (1973)).

The vector may contain at least one positive marker that enables the selection of cells carrying the inserted nucleic acids. The selectable molecule may be a gene which, upon introduction into the host cell, expresses a dominant phenotype permitting positive selection of cells carrying the gene ex vivo. Genes of this type are known in the art and include, for example, drug resistance genes such as hygromycin-B phosphotransferase (hph) which confers resistance to the antibiotic G418; the aminoglycoside phosphotransferase gene (*neo* or *aph*) from Tn5 which codes for resistance to the antibiotic G418; the dihydrofolate reductase (DHRF) gene; the

adenosine deaminase gene (ADA) and the multi-drug resistance (MDR) gene.

Recombinant viral vectors are introduced into host cells using standard techniques. Infection techniques have been developed which use recombinant infectious virus particles for gene delivery into cells. Viral vectors used in this way include vectors derived from simian virus 40 (SV40; Karlsson et al., *Proc. Natl. Acad. Sci. USA* 82:158 (1985)); adenoviruses (Karlsson et al., *EMBO J.* 5:2377 (1986)); vaccinia virus (Moss et al., *Vaccine* 6:161-3 (1988)); and retroviruses (Coffin, in Weiss et al. (Eds.), RNA Tumor Viruses, 2nd Ed., Vol. 2, Cold Spring Laboratory, NY, pp. 17-71 (1985)).

Nonreplicating viral vectors can be produced in packaging cell lines which produce virus particles which are infectious but replication defective, rendering them useful vectors for introduction of nucleic acid into a cell lacking complementary genetic information enabling encapsidation (Mann et al., *Cell* 33:153 (1983); Miller and Buttimore, *Mol. Cell. Biol.* 6:2895 (PA317, ATCC CRL9078). Packaging cell lines which contain amphotrophic packaging genes able to transduce cells of human and other species origin are preferred.

Vectors containing the inserted inhibitor genes or coding sequences are introduced into host cell using standard methods of transfection including electroporation, liposomal preparations, Ca-PH-DNA gels, DEAE-dextran, nucleic acid particle "guns" and other suitable methods.

In additional to various vectors including viral vectors, other delivery systems may be used including, but not limited to, microinjection (DePamphilis et al., *BioTechnique* 6:662-680 (1988)); liposomal mediated transfection (Felgner et al., *Proc. Natl. Acad. Sci. USA* 84:7413-7417 (1987); Felgner and Holm, *Focus* 11:21-25 (1989) and Felgner et al., *Proc. West. Pharmacol. Soc.* 32:115-121 (1989)); use of naked or particle mediated DNA transfer and other methods known in the art. Recently, cationic liposomes have been used to enhance transfection (Felgner et al., *Nature* 349:351 (1991); Zhu et al., *Science* 261:209 (1993)).

Suitable host cells for gene transfer consist of vertebrate cells such as fibroblasts, keratinocytes, muscle cells, mesangial cells (see, Kitamura et al., *Kidney Int.* 48:1747-1757 (1995)), and any other suitable host cell including so-called universal host cells, i.e. cells obtained from a different donor than the recipient subject but genetically modified to inhibit rejection by the subject. Autologous cells are preferred, but heterologous cells are encompassed within the scope of the invention.

Expression of the selected TGFβ inhibitor genes after introduction into the host cells is confirmed using standard methods. For example, expression of TGFβ-specific inhibitory agents can be determined by assaying for the ability of the supernatant from transfected cells to inhibit the binding of radiolabeled TGFβ to human mesangial cells using Fluorescent Activated Cell Sorting (FACS) or ELISA. Expression from host cells of an agent that inhibits TGFβ indirectly, such as Losartan, can be confirmed by detecting a decrease in fibronectin production by mesangial cells exposed to supernatant from transfected cells, relative to controls. Expression of genes encoding ECM degrading agents can be determined using, for example, an in vitro system using mesangial cells cultured on a ECM substrate such as Matrigel™ (Collaborative Research, Inc., Bedford, MA) that contains the major components of the mesangial matrix, including laminin, type IV collagen, entactin and heparan sulfate proteoglycan, as described by Baricos et al., *Kidney Internatl.* 47:1039-1047 (1995)). The ECM substrate is radiolabeled. and ECM degradation by the product of an expressed gene from transfected host cells is determined by measuring the release of radioactivity from the ECM into serum-free medium. These assay systems may also be employed to screen candidate TGFβ inhibiting and ECM degrading agents.

Administration of TGFβ Inhibitory Agents and Agents Degrading Accumulated ECM

Agents for inhibiting TGFβ and agents for degrading accumulated ECM are suspended in physiologically compatible pharmaceutical carriers, such as physiological saline, phosphate-buffered saline, or the like to form physiologically acceptable aqueous pharmaceutical compositions for administration to a subject. Parenteral vehicles include sodium chloride solution, Ringer's desctrose, dextrose and sodium chloride and lactated Ringer's solution. Other substances may be added a desired, such as antimicrobials.

The TGFβ inhibiting and ECM degrading agents may be administered together or apart, simultaneously or sequentially, to carry out the methods of the invention.

Modes of administration of the TGFβ inhibitory agents and ECM degrading agents are those known in the art for therapeutic agents and include parenteral, for example, intravenous (e.g. for antibody inhibitors or proteases), intraperitoneal, intramuscular, intradermal, and epidermal including subcutaneous and intradermal, oral (e.g. small molecule renin and TGFβ antagonists), or applied to mucosal surfaces, e.g. by intranasal administration using inhalation of aerosol suspensions, and by implanting to muscle or other tissue in the subject (e.g. for gene transfer of nucleic acid expressing renin and/or TGFβ inhibitors). Suppositories and topical preparations are also contemplated.

The TGFβ inhibitory and ECM degrading agents are introduced in amounts sufficient to prevent or reduce excess accumulation of extracellular matrix in susceptible tissues and organs including, but not limited to, lung and kidney tissue. Before or after administration, if necessary to prevent or inhibit the subject's immune response to the vehicles carrying the inhibitors, immunosuppressant agents may be used. Alternatively, the vehicles carrying the TGFβ inhibitory and ECM degrading agents can be encapsulated.

The most effective mode of administration and dosage regimen for the TGFβ inhibitory and ECM degrading agents for use in the methods of the present invention depend on the extent of TGFβ overproduction, the severity of the accumulation of extracellular matrix and resulting impairment of tissue or organ function, the subject's health, previous medical history, age, weight, height, sex and response to treatment and the judgment of the treating physician. Therefore, the amount of TGFβ inhibitory and ECM degrading agents to be administered, as well as the number and timing of subsequent administrations, are determined by a medical professional conducting therapy based on the response of the individual subject. Initially, such parameters are readily determined by skilled practitioners using appropriate testing in animal models for safety and efficacy, and in human subjects during clinical trials of candidate therapeutic formulations. Suitable animal models of human fibrotic conditions are known (see, e.g. Border and Noble, *New Eng. J. Med.* 331:1286-1292 (1994), incorporated by reference

herein).

After administration, the efficacy of the therapy using the methods of the invention is assessed by various methods including biopsy of kidney, lung or liver or other tissue to detect the amount of extracellular matrix accumulated. An absence of significant excess accumulation of ECM, or a decrease in the amount or expansion of ECM in the tissue or organ will indicate the desired therapeutic response in the subject. Preferably, a non-invasive procedure is used to detect a therapeutic response. For example, changes in TGFβ activity can be measured in plasma samples taken before and after treatment with an inhibitor (see, Eltayeb et al., *J. Am. Soc. Nephrol.* 8:110A (1997)), and biopsy tissue can be used to individually isolate diseased glomeruli which are then used for RNA isolation. mRNA transcripts for TGFβ, and extracellular matrix components (e.g. collagen) are then determined using reverse transcriptase-polymerase chain reaction (RT-PCR) (Peten et al., *J. Exp. Med.* 176:1571-1576 (1992)).

Advantages of the Invention

The invention provides improved treatment and prevention of fibrotic conditions associated with overproduction of TGFβ and excess accumulation of ECM in tissues and/or organs resulting in impaired function, or scarring, by reducing TGFβ overproduction directly and that resulting from multiple biological pathways, to effectively inhibit the TGFβ induced component of extracellular matrix deposition, and by increased degradation of ECM using degrading agents.

The therapeutic effects of the invention result from a reduction in or prevention of the TGFβ-induced excess accumulation of extracellular matrix in tissues and/or organs, and when combined with ECM degrading agents, from the increased degradation of ECM over time.

The following examples are presented to demonstrate the methods of the present invention and to assist one of ordinary skill in using the same. The examples are not intended in any way to otherwise limit the scope of the disclosure of the protection granted by Letters Patent granted hereon.

EXAMPLE I

DEMONSTRATION THAT RENIN UPREGULATES TGFβ IN HUMAN MESANGIAL CELLS

Normal fetal human mesangial cells (Clonetics Corp., Clonetics, Walkersville, MD) passaged 5 to 8 times, were plated (3,000 cell/cm^2) in 12 well plates in 2ml of medium (Mesangial Basal Medium (Clonetics Corp.) containing 5% FCS, 10 μg/ml penicillin and 100 μg/ml streptomycin) and allowed to grow to confluence for 48 hours at 37°C, 5% CO_2. Cultures were washed three times using sterile phosphate buffered saline at room temperature and then 2 ml/well of serum free MBM medium to induce quiescence. After 48 hours, the serum-free medium was removed and 2 ml/well of fresh serum-free medium was added. Human recombinant renin (Hoffman-La Roche Ltd., Basel, Switzerland) in concentrations from 10^{-6} to 10^{-12} M was added to each well. A blank and 5 ng/ml of TGFβ (R & D Systems, Minneapolis, MN) were used as controls. Cells and supernatants were harvested by centrifugation after 24 hrs of culture and frozen at -70°C until analysis. The total production and release of TGFβ into the culture supernatant was measured using an ELISA kit (R & D Systems). Induction of PAI-1 and fibronectin in the supernatant are also measured using anti-PAI-1 and anti-fibronectin antibodies in an ELISA to provide further confirmation of the inhibition of TGFβ. TGFβ, fibronectin and PAI-1 mRNA are measured using semi-quantitative RT-PCR.

(1) Determination of Dose Dependency of Renin Induction of TGFβ

As shown in Figure 2, renin increases the TGFβ production by cultured human mesangial cells in a dose-dependent manner.

EXAMPLE II

DEMONSTRATION OF THE EFFECT OF INHIBITING RENIN ON TGFβ PRODUCTION BY HUMAN MESANGIAL CELLS

Renin inhibitor Ro42-5892 (Hoffman-LaRoche, Basel, Switzerland), Losartan™ (Merck Pharmaceuticals, West Point, PA), Enalapril™ (Sigma Chemical Co., St. Louis, MO, Prod. No. E6888), or TGFβ1 neutralizing antibody (R & D Systems) were added in the amounts indicated below to separate wells in triplicate to block the renin cascade at different sites after stimulation by renin:

10^{-5} M Renin Inhibitor R042-5892 (Hoffman-LaRoche)
30 ng/ml Anti-TGFβ1 antibody (R & D Systems, #AB 101 NA)
30 ng/ml Chicken IgG (control for anti-TGFβ1 antibody, R & D Systems, # AB 101 C)
10^{-5} M Enalapril™ (Sigma Chemical Co., St. Louis, MO)
10^{-5} M Losartan™ (Merck Pharmaceuticals, West Point, PA)

These inhibitors were added at zero time with 10^{-7} M human recombinant renin (Hoffman-LaRoche).

As shown in Figure 3, use of inhibitors that block renin's action to increase Angiotensin II, i.e. blocking Angiotensin I production from Angiotensinogen (Ro 42-5892), blocking Angiotensin I conversion to Angiotensin II (Enalapril™) and blocking binding of Angiotensin II to its type I receptor (Losartan™), does not reduce the renin-induced increase in TGFβ production. These results demonstrate for the first time an alternative pathway in which TGFβ production is stimulated by renin.

EXAMPLE III

DEMONSTRATION OF INHIBITION OF TGFβ BY BLOCKING RENIN IN VIVO IN THE PRESENCE OF AN ANTI-FIBROTIC DRUG

In this example, a known fibrotic disease drug, Enalapril™ which inhibits the production of Angiotensin II, is combined with an inhibitor of renin, antisense renin oligonucleotide, to obtain an enhanced therapeutic effect on fibrotic disease in an animal model.

Rats are administered Enalapril™ in their drinking water prior to anti-thymocyte serum injection, e.g. three (3) days prior to injection. Anti-thymocyte antibody, e.g. OX-7, is injected intravenously into the rats at day three to produce fibrotic disease. (Bagchus et al., Lab. Invest. 55:680-687 (1986)). Renin antisense oligonucleotides are administered one hour following administration of OX-7 by introducing the oligonucleotides into a suitable vehicle, such as HVJ liposomes, and injecting the formulations into the left renal artery of Sprague Dawley rats as described for renin genes by Arai et al., *Biochem. And Biophys. Res. Comm.* 206(2):525-532 (1995), incorporated by reference herein. A control consisting of nonsense encoding oligonucleotides (e.g. derived from the renin antisense gene sequence) is also injected into the left renal artery of additional rats. The renin antisense localizes in the juxtaglomerular apparatus of the glomerulus where renin is produced blocking renin production.

Animals are sacrificed on day 7 and kidney tissue samples are taken for analysis of levels of TGFβ in the glomeruli. Glomeruli are sieved individually from each rat and placed in culture in suitable medium for three days. At the end of culture, culture supernatant is harvested by centrifugation and TGFβ, fibronectin and PAI-1 production are determined as markers of fibrotic renal disease severity. Other glomeruli are pooled and used to isolate RNA. RNA is used by standard methods to quantitate expression of mRNAs of interest, including TGFβ, fibronectin and collagens.

Glomeruli are also examined histologically for phenotypical changes, e.g. changes resulting from deposition for ECM. Phenotypic changes are associated with pathological

alteration of glomeruli indicative of fibrotic disease. Such changes include expansion of extracellular matrix in the mesangial area of the kidney in animal models and the presence of activated mesangial cells which have acquired the characteristics of fibroblasts, e.g. expressing α-smooth muscle actin and interstitial collagen, indicating progressive glomerular injury (Johnson et al., *J. Am. Soc. Nephrol.* 2:S190-S197 (1992)). Tissue for light microscopy is fixed in formaldehyde, then dehydrated in graded ethanol and embedded in paraffin. Sections are cut at 3 μm thickness and are stained with with the periodic Schiff reagent. The paraformaldehyde-fixed renal section of the rats are also incubated with mouse anti-human renin monoclonal antibody (Kaiichi Radioisotope Labs, Ltd., Tokyo, Japan), mouse anti-α-smooth muscle actin monoclonal antibody (Immunotech S. A. (Marseille, France) and rabbit anti-collagen antibodies (Chemicon, Temicula, CA, prod. No. AB755). The sections are further processed using Vectastain ABC Kit (Vector Laboratories, Inc., Burlingame, CA).

Results of antibody binding indicate the extent of glomerular injury and the effects of inhibition of renin on such injury.

EXAMPLE IV

ADDITIONAL DEMONSTRATION THAT RENIN UPREGULATES TGFβ IN HUMAN MESANGIAL CELLS

Primary cultures of adult human mesangial cells were grown from human nephrectomy tissues using standard methods. Cells were passaged 4-7 times and then plated (3,000 cell/cm^2) in 12 well plates in 2ml of medium (Mesangial Basal Medium (Clonetics Corp.) containing 5% FCS, 10 μg/ml penicillin and 100 μg/ml streptomycin) and allowed to grow to 70% confluency for 48 hours at 37°C, 5% CO_2. Cultures were washed three times using sterile phosphate buffered saline at room temperature and then 2 ml/well of serum free MBM medium to induce quiescence. After 48 hours, the serum-free medium was removed and 2 ml/well of fresh serum-free medium was added for 24 hours. Human recombinant renin (HrRenin, Hoffman-La Roche Ltd., Basel, Switzerland) in concentrations from 10^{-6} to 10^{-12} M was added to each well for 24 hours. A blank (no HrRenin) was used as a control. Cells and supernatants were harvested by

centrifugation after 24 hrs of culture and frozen at -70°C until analysis.

The total production and release of TGFβ into the culture supernatant was measured using an ELISA kit (R & D Systems). Induction of the matrix protein fibronectin (Fn) in the supernatant was measured using anti-fibronectin antibodies in an ELISA to provide further confirmation of induction of TGFβ. Renin-induced induction of TGFβ, fibronectin and PAI-1 mRNA were measured over time using semi-quantitative RT-PCR in a multiplex system where multiple cDNAs are amplified simultaneously according to Dostal et al., *Anal. Biochem.* 223:239-250 (1994), incorporated by reference herein Determinations were done in triplicate mesangial cell cultures.

(1) Determination of Dose Dependency of Renin Induction of TGFβ

As shown in Figure 4, statistically significant ($p < 0.05$) dose dependent increases in TGFβ (Figure 4A) and Fn production (Figure 4B) were observed, peaking with 2- and 1.4-fold increases at 10^{-6}M HrRenin, respectively. Time course experiments using 10^{-7}M HrRenin revealed significant increases in TGFβ and Fn production at 24 and 48 hours ($p<0.03$ (Figure 5A and B). As shown in Figure 6A-C, renin-induced increases in TGFβ, PAI-1 and Fn mRNAs peaked at 4 hours with increases from 1.5- to 2-fold.

(2) Demonstration that Renin Upregulation of TGFβ is not mediated through Renin Enzymatic Activity or Angiotensin II

Renin inhibitor Ro42-5892 (Hoffman-LaRoche, Basel, Switzerland), Losartan™ (Merck Pharmaceuticals, West Point, PA), Enalapril™ (Sigma Chemical Co., St. Louis, MO, Prod. No. E6888), or TGFβ1 neutralizing antibody (R & D Systems) were added in the amounts indicated below to separate wells in triplicate to block the renin cascade at different sites after stimulation by renin:

10^{-5} M Renin Inhibitor R042-5892 (Hoffman-LaRoche)

10^{-5} M Enalapril™ (Sigma Chemical Co., St. Louis, MO)

10^{-5} M Losartan™ (Merck Pharmaceuticals, West Point, PA)

Controls included neutralizing antibody to TGFβ (ATG) and control IgG (TgG)

These inhibitors were added at zero time with 10^{-7} M human recombinant renin (Hoffman-LaRoche).

As shown in Figure 7, use of inhibitors that block renin's action to increase Angiotensin II, i.e. blocking Angiotensin I production from Angiotensinogen (RO 42-5892), blocking Angiotensin I conversion to Angiotensin II (Enalapril™) and blocking binding of Angiotensin II to its type I receptor (Losartan™), does not reduce the renin-induced increase in TGFβ production.

These results provide additional evidence that renin upregulates TGFβ production by human mesangial cells through a mechanism which is independent of renin's enzymatic action to convert angiotensin to Angiotensin I, and independent of Angiotensin II generation. These results may have profound implications for progression of fibrotic renal disease, particularly in states of high plasma renin as are observed with therapeutic Angiotensin II blockade. Thus, the use of therapeutic agents such as Enalapril™ or Losartan™ for Angiotensin blockade may not be optimal as treatment agents because of resulting high renin levels, preventing a therapeutic reduction in TGFβ. In addition, antagonists developed to block the site on renin that acts in the Angiotensin II pathway, would not be expected to block the action of renin that is independent of this pathway. Therefore, effective therapy of fibrotic diseases must take these multiple pathways for TGFβ increase into consideration.

EXAMPLE V

DEMONSTRATION OF THE ABILITY OF tPA TO INCREASE PLASMIN DEGRADATION OF ACCUMULATED ECM IN VIVO

In this Example, recombinant tissue type plasminogen activator (rtPA) was shown to promote generation of the protease plasmin in nephritic glomeruli and to degrade pathological ECM proteins leading to a therapeutic reduction in matrix accumulation.

Six Sprague-Dawley rats with were injected with phosphate buffered saline (PBS, as a control) and 18 rats were injected with 300 ug of mouse monoclonal OX7 antibody produced in the laboratory using commercially obtained hybridoma cells (American Type Culture Collecton (Rockville, MD, USA; Peters et al., *Kidney Internatl.* 54:1570-1580 (1998)) on day 1 to induce anti-Thy-1 nephritis. Injection of the anti rat-thymocyte antibody intravenously causes binding to an epitope in rat glomerular mesangial cells call Thy 1.1. The complement-mediated mesangial cell lysis that follows initiates a cascade of tissue injury, followed by a repair process that involves induction of TGFβ-driven production and deposition of ECM components. In addition, the plasmin protease system is altered such that PA is decreased and PAI-1 is markedly increased. These alterations favor decreased plasmin generation which decreases matrix turnover and enhances matrix accumulation. Plasmin is the key to mesangial cell matrix turnover (Baricos et al, Kidney Int. 47:1037-1047 (1995)).

Three days after the initial injection, rtPA (Genentech, Inc., San Francisco, CA) in a formulation designed for rodent intravenous injection (GenBank EO8757) or PBS was injected intravenously. Injections were repeated twice a day from day 3 to day 5. RtPA was injected i.v. at a dose of 1 mg/kg BW (n=6). Controls received saline (n=6). Glomerular staining for ECM matrix proteins (collagen type I and III, fibronection EDA+ and tenascin) and glomerular mRNA levels of TGFβ1, fibronectin and PAI-1 were evaluated at day 6. Localization of rtPA in nephritic glomeruli and the effect of rtPA on glomerular plasmin were investigated. Rats were sacrificed at day 6 and kidney tissues excised, fixed in formalin and frozen for histological analysis.

Table 1

Groups of Six Rats	Treatment
Group 1-Normal controls	300 ug of PBS on day 1, then 300 ug PBS 2X
Group 2- Disease control	300 ug of OX7 on day 1, then 300 ug PBS 2X
Group 3 - Disease + Dose 1	300 ug of OX7 on day 1, then 0.25 mg/day rtPA 2X/day on days 3, 4 and 5

Kidney tissue sections were stained for extracellular matrix using Periodic Acid Schiff (PAS) using standard procedures and were stained for specific relevant matrix proteins such as Collagen I, Collagen IV, Fibronectin EDA and tenascin using standard immunohistochemical staining procedures. Matrix proteins were scored by image analysis of 30 glomeruli per rat.

Figure 8A (control) and B (tPA) show an overall decrease in matrix accumulated as a result of tPA treatment. Compared to the untreated, disease control group (Figure 9A-D), the percentage of the glomerular area with positive staining was significantly lower in the rtPA treated group at day 6 for fibronectin EDA+(FN) (19 ± 2 vs. 14 ± 1, $p<0.01$), laminin (35 ± 2 vs. 25 ± 2, $p<0.001$), type I collagen 33 ± 1 vs. 21 ± 3, $p<0.001$) and type IV collagen (27 ± 2 vs. 23 ± 1, $p<0.01$). Glomerular levels of TGFβ1, FN and PAI-1 mRNA were unchanged (Figure 10). rtPA co-localized with fibrin along the glomerular capillary loops and in the mesangium.

rtPA was injected into nephritic rats 10, 20 and 30 minutes before sacrifice. At sacrifice, glomeruli were isolated and placed in culture with a chromogenic substrate for tPA. Plasmin generation by nephritic glomeruli, as shown in Figure 11, was significantly elevated in tPA treated nephritic glomeruli compared to nephritic gomeruli from disease control rats.

This example demonstrates that injected rtPA binds fibrin in nephritic glomeruli where it increases plasmin generation and promotes pathological ECM degradation. rtPA may thus be used in the methods of the invention as an ECM degrading agent.

EXAMPLE VI

EFFECT OF ADMINISTRATION OF TGFβ INHIBITORY AGENTS AND AGENTS THAT PROMOTE DEGRADATION OF ECM

In this example, at least one agent that inhibits TGFβ, anti-TGFβ antibody or decorin, is administered in combination with an ECM degrading agent, such as rtPA to reduce excess ECM

accumulation and degrade accumulated ECM in an animal model of glomerulonephritis.

Sprague-Dawley rats are treated as described in the above Examples to induce nephritis. Groups of six (6) rats each include untreated disease controls, rats treated with tPA alone as in Example V, above, rats treated with Enalapril™ alone (200 mg/day) in drinking water and rats treated with both intravenous rtPA and Enalapril™ in drinking water. On day 6 rats are sacrificed and kidney sections are excised, fixed in formalin and frozen for histological analysis. Glomeruli are isolated and used for in vitro analysis of production of TGFβ, fibronectin and PAI-1 using ELISA assays of culture supernatants and for isolation of RNA for Northern analysis of message levels of TGFβ, fibronectin and PAI-1. Tissue samples are stained for ECM proteins and glomerular mRNA levels of TGFβ1, fibronectin and PAI-1.

It is expected that the results of treatments with both anti-TGFβ antibody and rtPA treatment are significantly lower positive staining both in PAS stained tissue and in glomeruli stained for specific matrix components, as shown in Example V, compared with groups treated with either agent alone or in the control disease group.

EXAMPLE VII

DEMONSTRATION OF THE EFECTS OF ADMINISTRATION OF A PAI-1 MUTANT ON EXTRACELLULAR MATRIX DEGRADATION

The human PAI-1 mutant used in this experiment (see WO 97/39028) was constructed on the wild-type PAI-1 background (Ginsburg et al., *J. Clin. Invest.* 78:1673-1680 (1986)), and disabled by the introduction of two Arg residues at positions 333 and 335 of the mature protein, which are also referred to as residues P14 and P12 of the reactive center loop (Lawrence, *Adv. Exp. Med. Biol.* 425:99-108 (1997)). Upon interaction with a proteinase, these substitutions greatly retard the insertion of the reactive center loop into β-sheet A and prevent the mutant from adopting the latent conformation. Since loop insertion results in loss of vitronectin affinity (Lawrence et al., 1997, supra), the PAI-1 mutant retains significant vitronectin activity while

failing to inhibit all plasminogen activators.

Four to six week old male Sprague-Dawley rats (Sasco, Inc., Omaha, NE) were treated as described in the above Examples to induce anti-thy-1 nephritis by intravenous injection of the monoclonal anti-thymocyte antibody OX-7 350 mg/200 g body weight. Groups of six (6) rats included a normal control group (injected with saline), an untreated disease control group (injected with PBS), and a group treated with 1 mg/Kg PAI-1 mutant injected once a day beginning 24 hours after induction of ATS nephritis and ending at day 5. Two additional groups of rats were treated with 1) 100 mg/liter of Enalapril (in drinking water) with a loading dose of Enalapril given by gavage 24 hr after disease induction followed by 100 mg/liter of Enalapril in drinking water, and 2) a 6% low protein diet (Teklad, Madison, WI, diet number TD86551) started 24 hours following disease induction.

Rats were sacrificed at day 6 and kidney tissues excised, fixed in formalin and frozen for histological analysis. Kidneys were perfused in situ with cold buffered saline (PBS) at pH 7.4, and then excised. Pieces of cortex were removed and either snap frozen in 2-methylbutane that had been cooled in liquid nitrogen or fixed in 10% neutralized formalin for immunohistologic examination. The capsules were removed and the cortical tissue dissected out and minced with a razor blade prior to isolation of glomeruli by standard graded seiving. Kidney tissue sections were stained for extracellular matrix using Periodic Acid Schiff (PAS) using standard procedures and were stained for specific relevant matrix proteins such as Collagen I, Collagen IV, Fibronectin EDA and tenascin using standard immunohistochemical staining procedures. Matrix proteins were scored by a blinded observer. 20 glomeruli per rat were evaluated. Isolated glomeruli were also used to determine glomerular mRNA levels of TGFβ1, fibronectin and PAI-1 at day 6.

Reagents to measure plasmin activity, including plasminogen, low molecular weight u-PA and H-D-Val-Leu-Lys-p-nitroanilide (S-2251) were obtained from KabiVitrum (Franklin, OH). PAI-1 activity was assayed by measuring the hydrolysis of synthetic substrate by formed plasmin in the presence of plasminogen (Marshall et al., *J. Biol. Chem.* 265:9198-8204 (1990)). Assays were performed in polyvinyl chloride microtiter plates. The total volume of 125 µl was

comprised of the following: sample, H-D-Val-Leu-Lys-P-nitroanilide (0.01 μM) and plasminogen (0.03 μM) in 0.5% Triton X-100, 0.1 M Tris, at pH 8.0. The amount of p-nitroaniline released was measured at 410 nm with a Thermomax microplate reader (Molecular Devices, Menlo Park, CA). A standard curve was generated with each assay using low molecular weight human u-PA. Each sample was also assayed without plasminogen to establish the plasminogen-dependence of the enzyme activity. The plasmin activity in culture supernatant or cell lysate was expressed as IU/1000 glomeruli.

Figure 12 shows an increase in plasmin generation of glomeruli in culture as a result of injection of the PAI-1 mutant. Compared to the untreated, disease control group, the glomerular plasmin activity was significantly higher in the PAI-1 treated group, being approximately halfway between the activity of disease controls and normal glomeruli. Notably, the significant increase in glomerular plasmin activity in nephritic glomeruli was observed with the PAI-1 mutant 24 hours following the final injection.

In addition, treatment with the PAI-1 mutant resulted in decreased accumulation of Collagen Type I, relative to diseases controls (Figure 13), while glomerular levels of TGFβ1, FN, PAI-1 mRNA and Collagen I mRNA were not significantly altered. The decreased accumulation of Collagen Type I together with the fact that the Collagen I mRNA does not significantly decrease suggests enhanced extracellular matrix degradation rather than decreased production of Collagen I.

These results suggest that the increase in glomerular plasmin activity with a PAI-1 mutant can be titrated to avoid large increases in plasmin generation that may lead to hemorrhaging. Thus, the dose of the PAI-1 mutant may be altered, for example by doubling the dose, to increase glomerular plasmin activity to normal, but not excessive, levels to decrease deleterious accumulation of extracellular matrix. In addition, the time of treatment may be extended, for example to 10 days to obtain desired degradation.

Various publications are cited herein that are hereby incorporated by reference in their entirety.

As will be apparent to those skilled in the art in which the invention is addressed, the present invention may be embodied in forms other than those specifically disclosed without departing from the spirit or potential characteristics of the invention. Particular embodiments of the present invention described above are therefore to be considered in all respects as illustrative and not restrictive. The scope of the invention is as set forth in the appended claims and equivalents thereof rather than being limited to the examples contained in the foregoing description.

WE CLAIM:

1. A method for treating a condition associated with the excess accumulation of extracellular matrix in a tissue and/or organ or at a dermal wound site comprising:

a) reducing the excess accumulation of extracellular matrix associated with TGFβ overproduction and/or activity in an organ or tissue, or at a wound site; and

b) degrading excess accumulated extracellular matrix in said tissue and/or organ or wound site;

whereby the accumulation of extracellular matrix in said tissue and/or organ or wound site is reduced from the level existing at the time of treatment.

2. The method of claim 1 wherein the accumulation of extracellular matrix is reduced to a level which does not interfere with normal functioning of the tissue or organ or result in scarring.

3. The method of claim 1 wherein said step of reducing the accumulation of extracellular matrix comprises administering at least one agent that inhibits TGFβ in an amount sufficient to inhibit TGFβ overproduction and/or activity.

4. The method of claim 3, wherein said agent that inhibits TGFβ is selected from the group consisting of selected from the group consisting of inhibitors of aldosterone, inhibitors of angiotensin II, anti-TGFβ antibodies, renin, ACE inhibitors, AII receptor antagonists, proteoglycans and ligands for the TGFβ receptor.

5. The method of claim 4 wherein said agent is a proteoglycan selected from the group consisting of decorin, biglycan, fibromodulin, lumican, betaglycan and endoglin.

6. The method of claim 4 wherein said ACE inhibitor is Enalapril™.

7. The method of claim 4 wherein said AII receptor antagonist is Losartan™.

8. The method of claim 1 wherein said step of reducing the accumulation of extracellular matrix associated with TGFβ activity comprises contacting renin with an anti-renin agent.

9. The method of claim 1 wherein said step of degrading excess accumulated extracellular matrix comprises contacting said matrix with at least one protease in an amount sufficient to degrade excess accumulated extracellular matrix to a level that does not impair the normal function of said tissue and/or organ or result in scarring.

10. The method of claim 9 wherein said protease is selected from the group consisting of serine proteases, metalloproteinases and protease combinations.

11. The method of claim 1 wherein said step of degrading accumulated extracellular matrix comprises administering an agent which increases the amount of active protease sufficient to degrade excess accumulated matrix to a level that does not impair the normal function of said tissue and/or organ or result in scarring.

12. The method of claim 11 wherein said protease is selected from the group consisting of serine proteases, metalloproteinases and protease combinations.

13. The method of claim 12 wherein said protease is plasmin and said agent which increases the amount of active plasmin is tPA.

14. The method of claim 12 wherein said protease is plasmin and said agent which increases the amount of active plasmin is a PAI-1 mutant.

15. The method of claim 1 wherein said condition associated with the excess accumulation of extracellular matrix is a fibrotic condition.

16. The method of claim 15 wherein said fibrotic condition is selected from the group consisting of glomerulonephritis, adult or acute respiratory distress syndrome (ARDS), diabetes, diabetic kidney disease, liver fibrosis, kidney fibrosis, lung fibrosis, post infarction cardiac fibrosis, fibrocystic diseases, fibrotic cancer, post myocardial infarction, left ventricular hypertrophy, pulmonary fibrosis, liver cirrhosis, veno-occlusive disease, post-spinal cord injury, post-retinal and glaucoma surgery, post-angioplasty restenosis, renal interstitial fibrosis, arteriovenous graft failure and scarring.

17. The method of claim 1 wherein said tissue or organ is selected from the group consisting of kidney, lung, liver, heart, arteries, skin and the central nervous system.

18. The method of claim 1 wherein said condition associated with the excess accumulation of extracellular matrix is scarring.

19. The method of claim 3 wherein said agent that inhibits TGFβ is nucleic acid encoding the agent.

20. The method of claim 9 wherein said protease is nucleic acid encoding a protease.

21. A method for treating a condition associated with the excess accumulation of extracellular matrix in a tissue and/or organ or at a wound site comprising:

a) administering at least one agent that inhibits TGFβ to reduce additional accumulation of extracellular matrix associated with TGFβ activity in a tissue and/or organ or at a wound site; and

b) administering at least one agent to degrade excess accumulated extracellular matrix; whereby the accumulation of extracellular matrix in said tissue and/or organ or at said wound site is reduced to a level which does not interfere with normal functioning of the tissue or organ in which said extracellular matrix accumulated.

22. The method of claim 21 wherein said agent that inhibits TGFβ and said agent to degrade excess accumulated extracellular matrix are administered concurrently.

23. The method of claim 21 wherein said agent that inhibits TGFβ and said agent to degrade excess accumulated extracellular matrix are administered sequentially.

24. The method of claim 21 wherein the agent that inhibits TGFβ is an anti-TGFβ specific agent that binds to and inhibits the activity of TGFβ.

25. The method of claim 21 wherein the agent that inhibits TGFβ is an agent selected from the group consisting of inhibitors of aldosterone, inhibitors of angiotensin II, anti-TGFβ antibodies, renin, ACE inhibitors, AII receptor antagonists, proteoglycans and ligands for the TGFβ receptor.

26. A method for treating or preventing a condition associated with the excess accumulation of extracellular matrix in a tissue and/or organ, or at a wound site, comprising administering a combination of agents in an amount sufficient to inhibit TGFβ activity and/or production to prevent or reduce the excess accumulation of extracellular matrix associated with TGFβ overproduction in a tissue and/or organ, or at a wound site.

27. The method of claim 26 wherein the agents to inhibit TGFβ comprise an agent that binds to and inhibits the activity of TGFβ and an agent selected from the group consisting of inhibitors of aldosterone, inhibitors of angiotensin II, inhibitors of renin, ACE inhibitors and AII receptor antagonists.

28. The method of claim 27 wherein said agent that binds to and inhibits the activity of TGFβ is selected from the group consisting of anti-TGFβ antibodies, proteoglycans and ligands for the TGFβ receptor.

29. The method of claim 28 wherein said agent that binds to and inhibits the activity of TGFβ is a proteoglycan selected from the group consisting of decorin, biglycan, fibromodulin, lumican, betaglycan and endoglin.

30. The method of claim 26 wherein said agents are nucleic acids encoding said respective agents.

31. The method of claim 26 wherein said agents are administered to reduce TGFβ overproduction prior to excess accumulation of extracellular matrix.

32. A method for preventing or reducing excess extracellular matrix accumulation in a tissue or organ or at a wound site comprising inhibiting the overproduction of TGFβ present in an organ or tissue or at a wound site to prevent

33. The method of claim 32 further comprising the step of degrading excess accumulated extracellular matrix in said tissue and/or organ or at said wound site.

34. The method of claim 32 wherein said accumulation of extracellular matrix is reduced to a level which does not interfere with the normal functioning of the tissue and/or organ in which said extracellular matrix accumulated or scarring is prevented or reduced.

35. A composition for reducing TGFβ overproduction in a tissue and/or organ or at a wound site comprising a combination of agents that inhibit TGFβ activity and/or production in a pharmaceutically acceptable carrier.

36. The composition of claim 35 wherein said agents comprise an agent that binds to and inhibits TGFβ and an agent that inhibits a pathway resulting in TGFβ production.

37. A composition for preventing or reducing excess accumulation of extracellular matrix associated with TGFβ activity comprising at least one agent that inhibits TGFβ activity and/or production and at least one agent that degrades excess accumulated extracellular matrix in a pharmaceutically acceptable carrier.

38. The composition of claim 37 wherein said agent that inhibits TGFβ is an agent that binds to and inhibits TGFβ, and said agent that degrades excess accumulated extracellular matrix is a protease.

39. The composition of claim 38 wherein said agent that inhibits TGFβ is selected from the group consisting of inhibitors of aldosterone, inhibitors of Angiotensin II, anti-TGFβ antibodies, renin, ACE inhibitors, AII receptor antagonists, proteoglycans and ligands for the TGFβ receptor.

40. The composition of claim 38 wherein said protease is selected from the group consisting of serine proteases, metalloproteinases and protease combinations.

41. The composition of claim 39 wherein said proteoglycan is selected from the group consisting of decorin, biglycan, fibromodulin, lumican, betaglycan and endoglin.

42. The composition of claim 37 wherein said agent that inhibits TGFβ and said agent that degrades excess accumulated extracellular matrix are nucleic acids encoding said agents respectively.

43. The composition of claim 37 wherein said agent that degrades excess accumulated extracellular matrix is an agent that increases the amount of active protease present in the tissue and/or organ or at a wound site.

44. The composition of claim 43 wherein said protease is plasmin and said agent is tPA.

45. The composition of claim 43 wherein said protease is plasmin and said agent is a PAI-1 mutant.

46. A composition for reducing the excess accumulation of extracellular matrix in a tissue and/or organ, or at a dermal wound site, comprising an agent that increases the amount of active protease present in the tissue and/or organ or at the wound site in a pharmaceutically acceptable carrier.

47. The composition of claim 46 wherein said protease is plasmin and said agent is tPA.

48. The composition of claim 46 wherein said protease is plasmin and said agent is a PAI-1 mutant.

49. A composition for treating a condition associated with the excess accumulation of extracellular matrix in a tissue and/or organ or at a dermal wound site comprising a combination of agents that inhibit TGFβ in a pharmaceutically acceptable carrier.

50. The composition of claim 49 wherein said agents that inhibit TGFβ are selected from the group consisting of inhibitors of aldosterone, inhibitors of inhibitors of aldosterone, inhibitors of angiotensin II, anti-TGFβ antibodies, renin, ACE inhibitors, AII receptor antagonists, proteoglycans and ligands for the TGFβ receptor.

51. The composition of claim 50 wherein said proteoglycans are selected from the group consisting of decorin, biglycan, fibromodulin, lumican, betaglycan and endoglin.

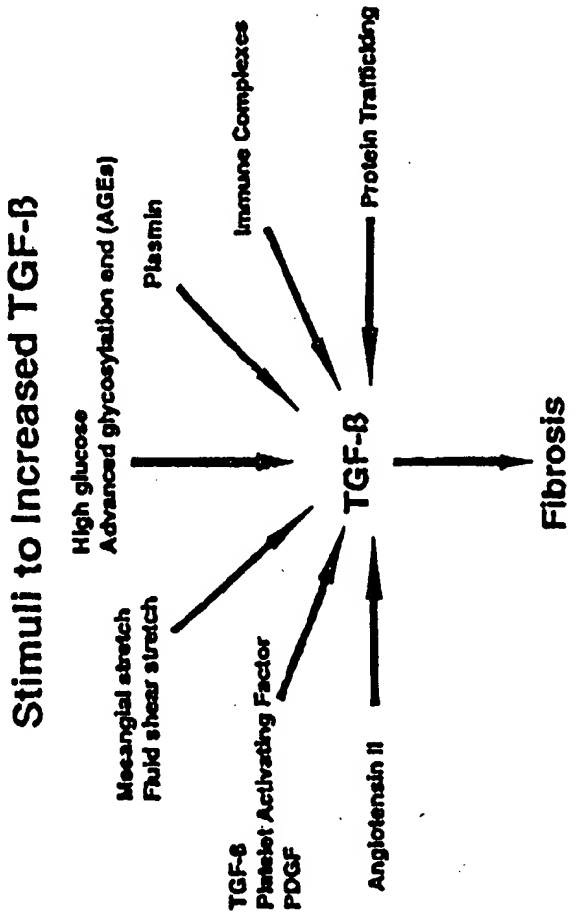

Stimuli to Increased TGF-β
High glucose
Advanced glycosylation end (AGEs)
Plasmin
Immune Complexes
Protein Trafficking
Mesangial stretch
Fluid shear stretch
TGF-β
Platelet Activating Factor
PDGF
Angiotensin II
Fibrosis

FIGURE 1

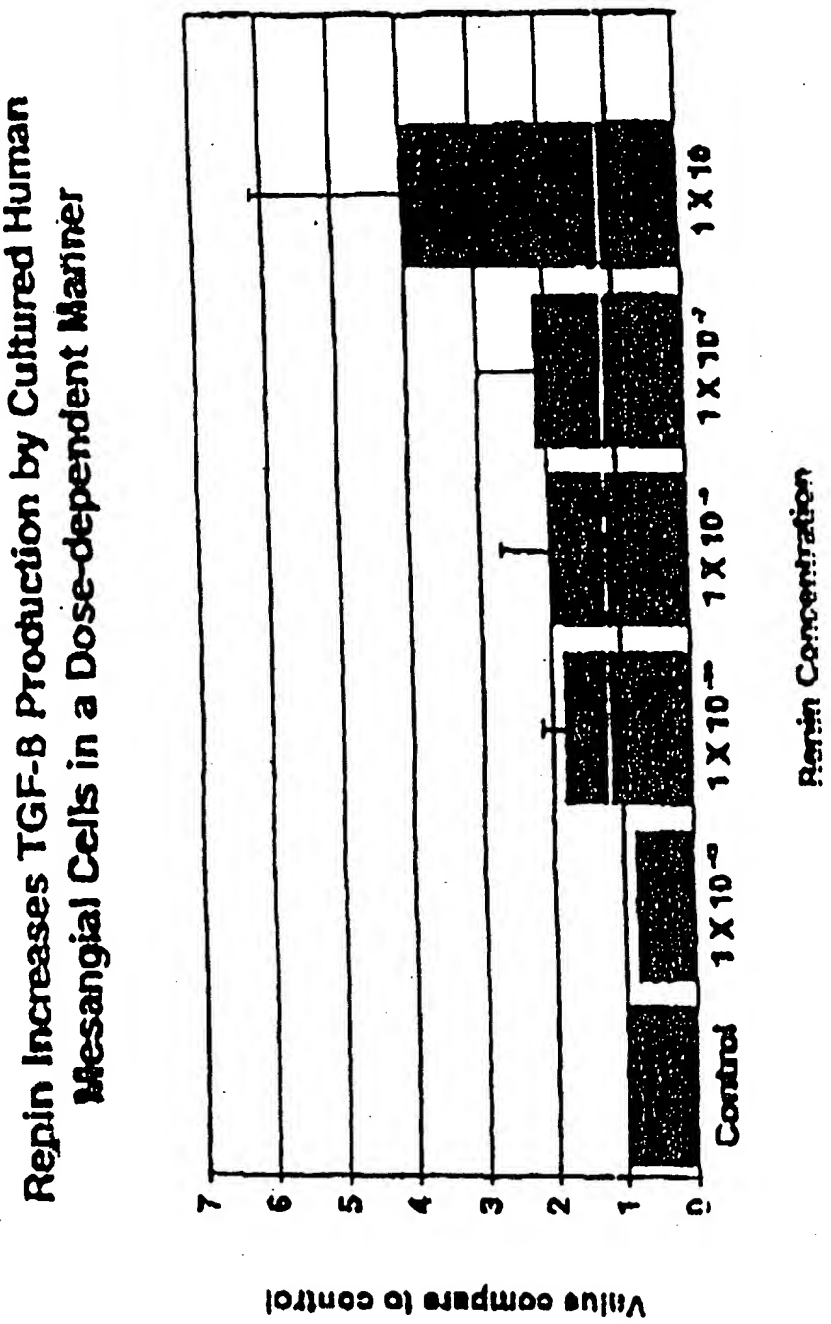

Renin Increases TGF-B Production by Cultured Human Mesangial Cells in a Dose-dependent Manner
Value compare to control
0
1
2
3
4
5
6
7
Control
1 X 10⁻⁸
1 X 10⁻⁸
1 X 10⁻⁷
1 X 10⁻⁷
1 X 10
Renin Concentration

FIGURE 2

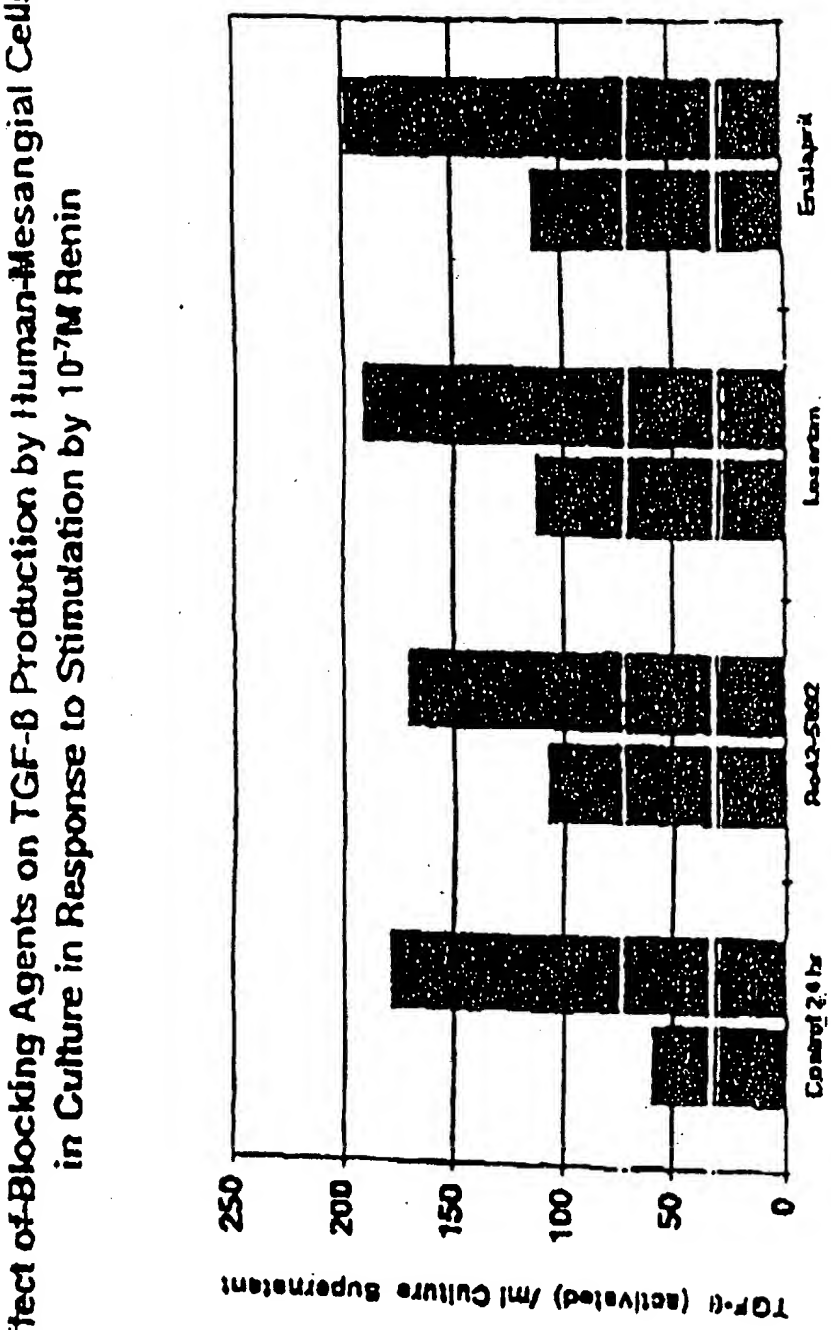
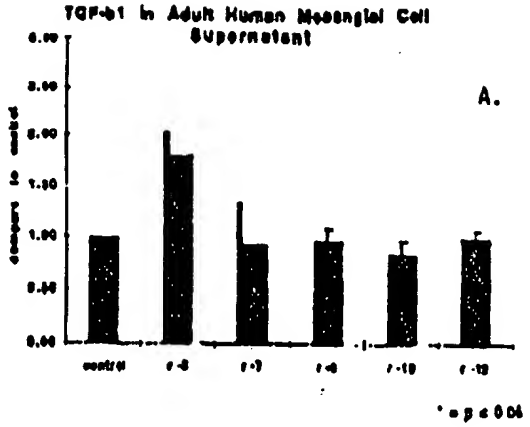

Effect of Blocking Agents on TGF-β Production by Human Mesangial Cells in Culture in Response to Stimulation by 10^{-7}M Renin
TGF-β (activated) /ml Culture Supernatant
250
200
150
100
50
0
Control 24 hr
Ro42-5892
Losartan
Enalaprilat

FIGURE 3


TGF-b1 in Adult Human Mesangial Cell Supernatant
A.
control
t -5
t -7
t -9
t -10
t -12

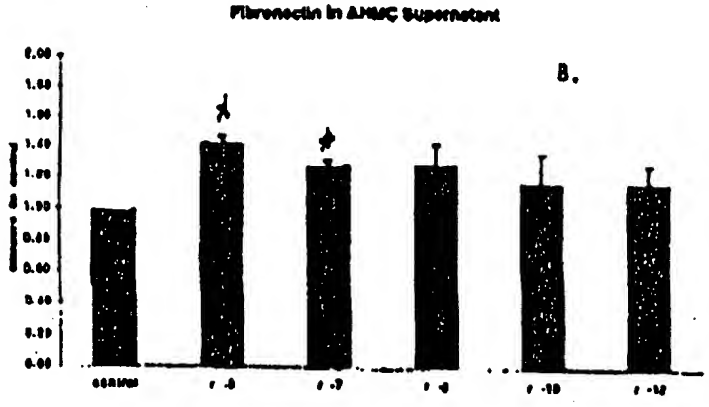
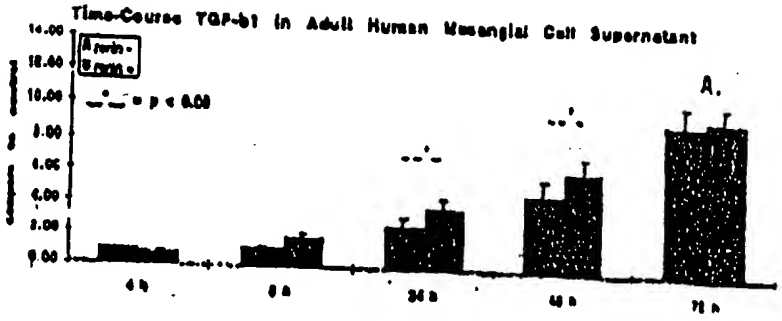

Fibronectin in AMSC Supernatant
B.
control
t -5
t -7
t -9
t -10
t -12

FIGURE 4


Time-Course TGF-b1 in Adult Human Mesangial Cell Supernatant
A.
4 h
8 h
24 h
48 h
72 h

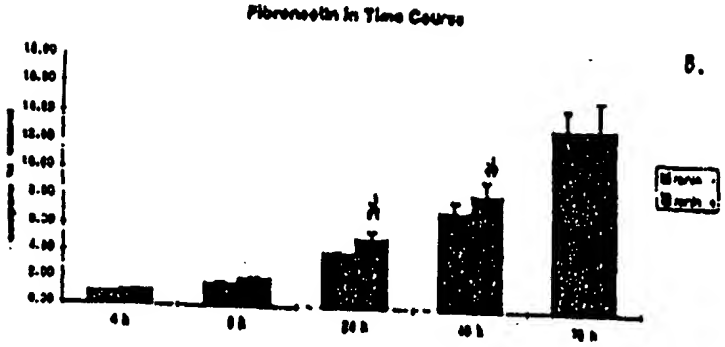

Fibronectin in Time Course
B.
4 h
8 h
24 h
48 h
72 h

FIGURE 5

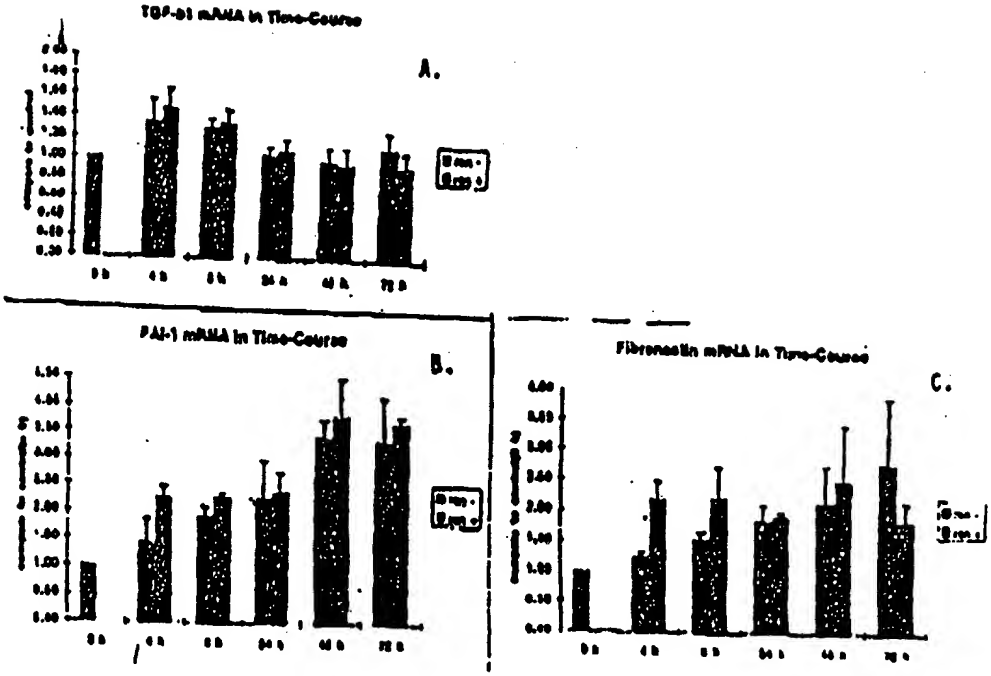


FIGURE 6

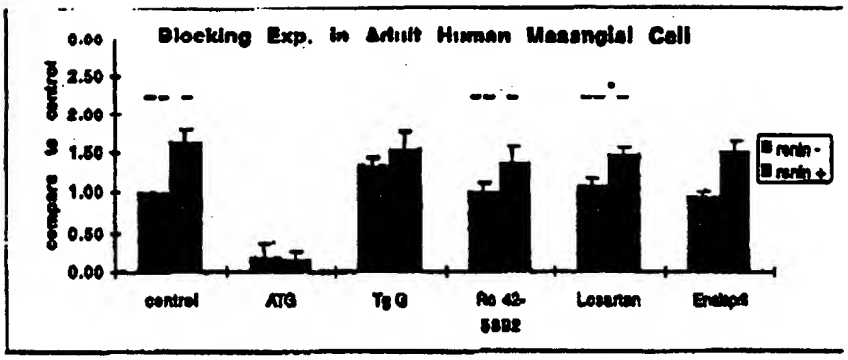

Blocking Exp. in Adult Human Mesangial Cell
compare to control
0.00
2.50
2.00
1.50
1.00
0.50
0.00
renin -
renin +
control
ATG
Tg G
Ro 42-
5892
Losartan
Enalapril

FIGURE 7

Effect of tPA on anti-Thy-1 nephritis at day 6

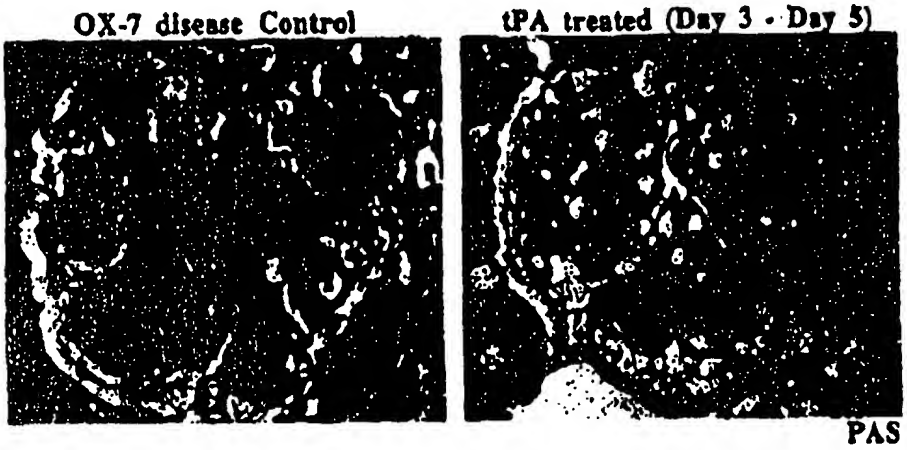


FIGURE 8

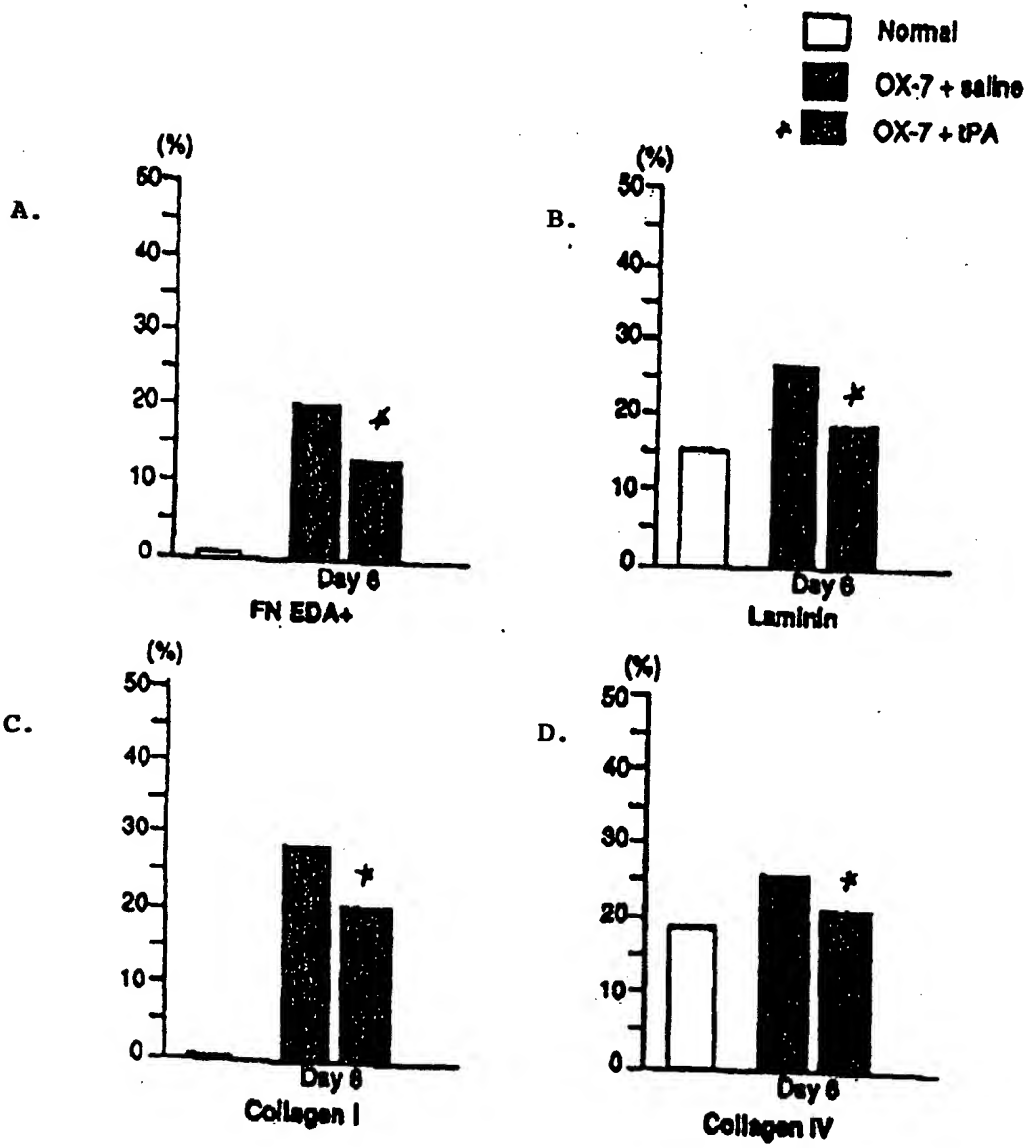

Staining Index (%)
(percentage of positive staining area/glomerular area)
Normal
OX-7 + saline
OX-7 + tPA
A.
B.
C.
D.
(%)
50
40
30
20
10
0
*
Day 6
FN EDA+
Laminin
Collagen I
Collagen IV

FIGURE 9

Effect of tPA on glomerular mRNA expressions at Day 6

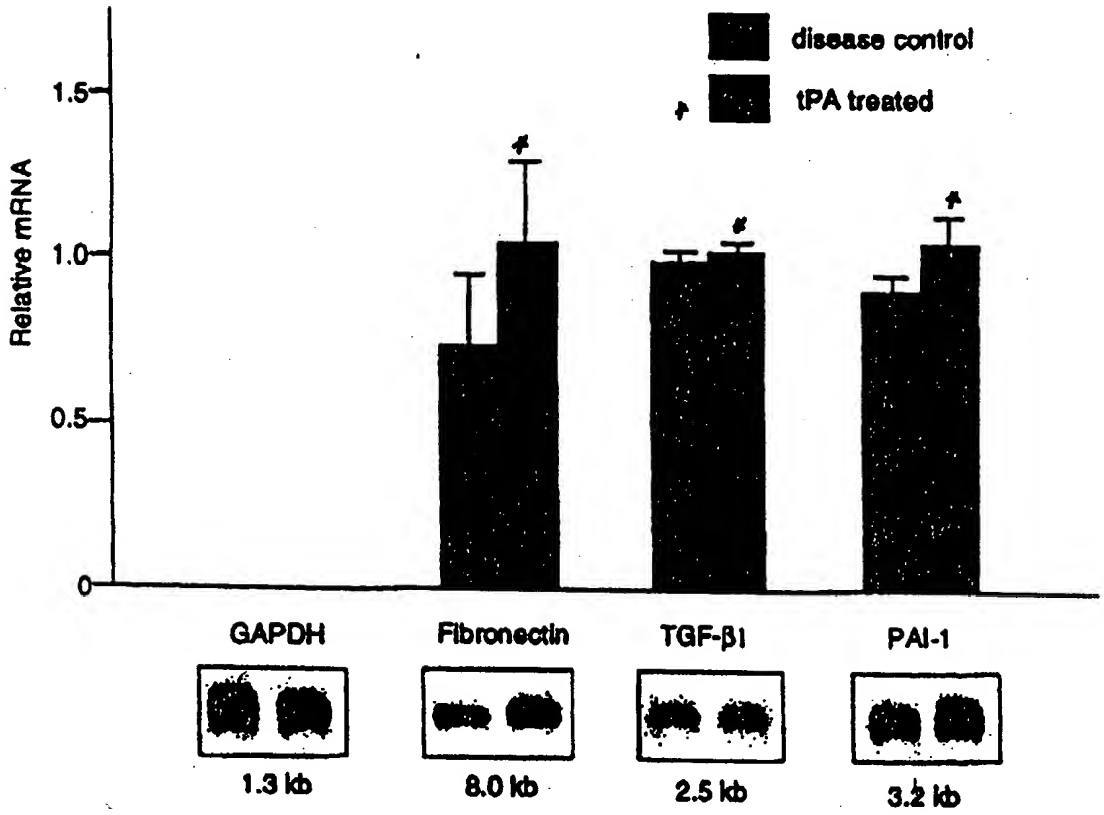
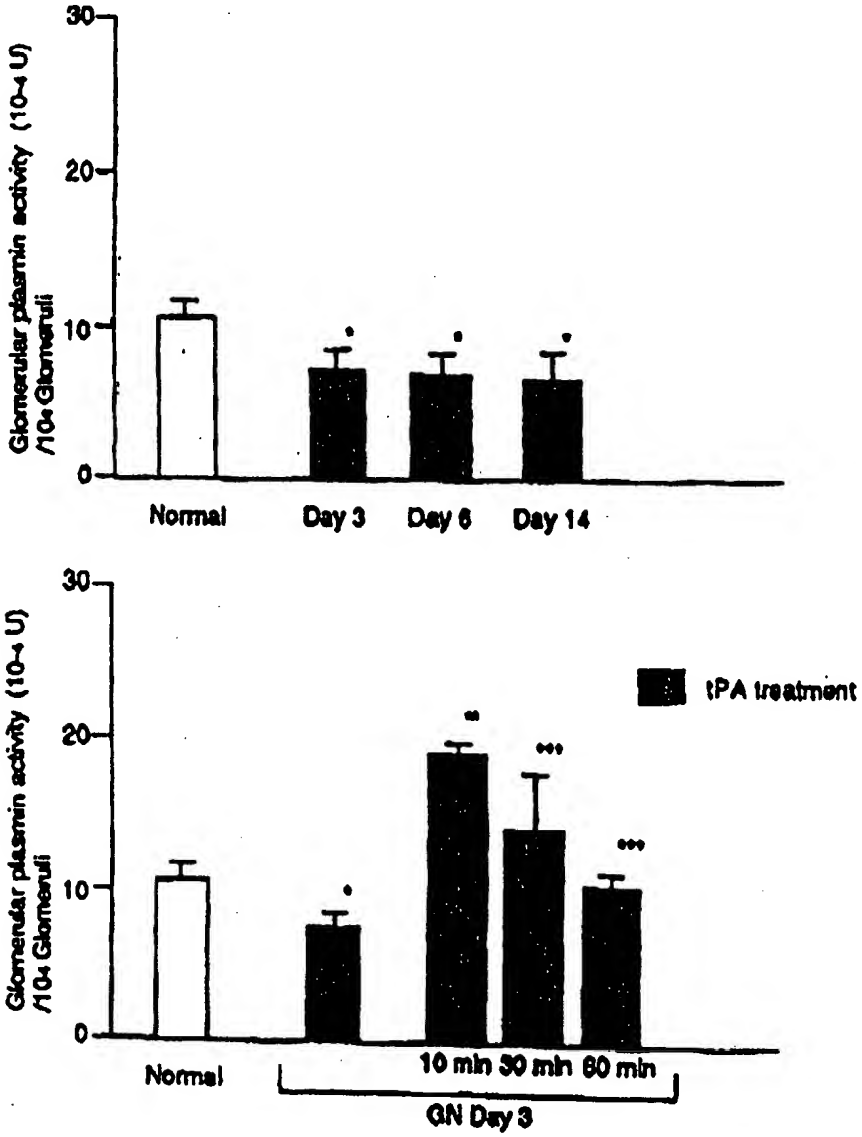


FIGURE 10

Plasmin activity of isolated glomeruli



*P<0.01 vs. normal
**P<0.01 vs. disease control
***P<0.05 vs. disease control

FIGURE 11

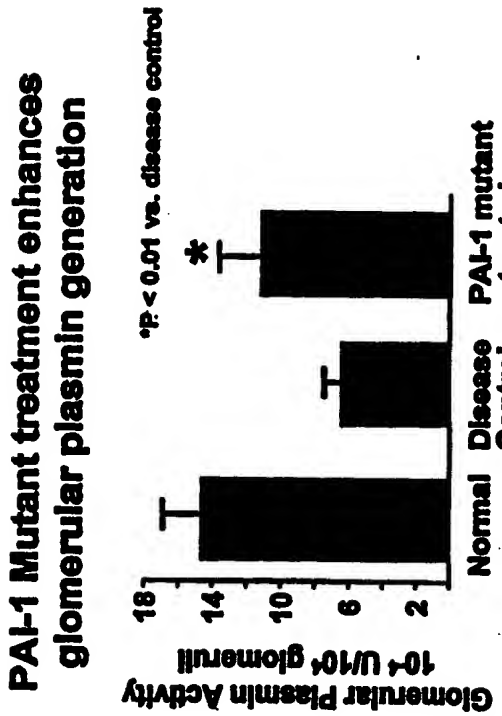

PAI-1 Mutant treatment enhances glomerular plasmin generation
*P < 0.01 vs. disease control
Glomerular Plasmin Activity
10⁻⁴ U/10⁴ glomeruli
18
14
10
6
2
*
Normal
Disease Control
PAI-1 mutant treated
Treatment Group

FIGURE 12

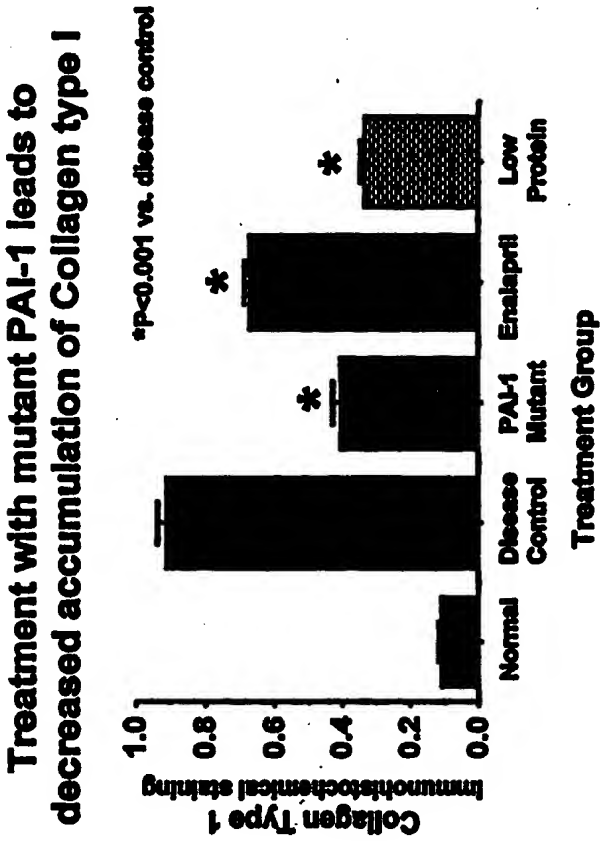

Treatment with mutant PAI-1 leads to decreased accumulation of Collagen type I
*P<0.001 vs. disease control
Collagen Type 1 immunohistochemical staining
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
Normal
Disease Control
PAI-1 Mutant
Enalapril
Low Protein
Treatment Group

FIGURE 13

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
13 July 2000 (13.07.2000)

PCT

(10) International Publication Number
WO 00/40227 A3

(51) International Patent Classification[7]: A61K 31/00, A61P 13/12

(21) International Application Number: PCT/US00/00179

(22) International Filing Date: 5 January 2000 (05.01.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/114,795 5 January 1999 (05.01.1999) US

(71) Applicants *(for all designated States except US)*: **UNIVERSITY OF UTAH** [US/US]; 615 Arapeen Drive, Suite 110, Salt Lake City, UT 84108 (US). **AMERICAN NATIONAL RED CROSS** [US/US]; 15601 Crabbs Branch Way, Rockville, MD 20855 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **NOBLE, Nancy, A.** [US/US]; 4464 South Abinadi, Salt Lake City, UT 84124 (US). **BORDER, Wayne, A.** [US/US]; 4464 South Abinadi, Salt Lake City, UT 84124 (US). **LAWRENCE, Daniel, A.** [US/US]; 16525 Keats Terrace, Derwood, MD 20855 (US).

(74) Agent: **MANDEL, Sara Lynn**; Mandel & Adriano, 35 No. Arroyo Parkway, Suite 60, Pasadena, CA 91103 (US).

(81) Designated States *(national)*: AL, AM, AT, AT (utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (utility model), DE, DE (utility model), DK, DK (utility model), EE, EE (utility model), ES, FI, FI (utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (utility model), SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

(88) Date of publication of the international search report:
18 January 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 00/40227 A3

(54) Title: METHODS FOR TREATING CONDITIONS ASSOCIATED WITH THE ACCUMULATION OF EXCESS EXTRACELLULAR MATRIX

(57) Abstract: The present invention is methods and compositions for reducing and preventing the excess accumulation of extracellular matrix in a tissue and/or organ or at a wound site using a combination of agents that inhibit TGFβ, or using agents that inhibit TGFβ in combination with agents that degrade excess accumulated extracellular matrix. The compositions and methods of the invention are used to treat conditions such as fibrotic diseases and scarring that result from excess accumulation of extracellular matrix, impairing tissue or organ function or skin appearance in a suject.

INTERNATIONAL SEARCH REPORT

Intern ıal Application No
PCT/US 00/00179

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/00 A61P13/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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P,X, Y	WO 99 34823 A (UNIV UTAH) 15 July 1999 (1999-07-15) the whole document ---	1-51
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X,Y	WO 91 04748 A (JOLLA CANCER RES FOUND) 18 April 1991 (1991-04-18) the whole document ---	1-51
X,Y	WO 93 09800 A (UNIV UTAH ;UNIV CALIFORNIA (US); JOLLA CANCER RES FOUND (US)) 27 May 1993 (1993-05-27) the whole document ---	1-51
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 November 2000	28/11/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Orviz Diaz, P

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X,Y	WO 93 10808 A (JOLLA CANCER RES FOUND) 10 June 1993 (1993-06-10) the whole document	1-51
X,Y	BORDER W A ET AL: "NATURAL INHIBITOR OF TRANSFORMING GROWTH FACTOR-SS PROTECTS AGAINSTSCARRING IN EXPERIMENTAL KIDNEY DISEASE" NATURE,GB,MACMILLAN JOURNALS LTD. LONDON, vol. 360, no. 6402, 1992, pages 361-364, XP000910224 ISSN: 0028-0836 the whole document	1-51
X,Y	BORDER W ET AL: "Interactions of transforming growth factor-beta and angiotensin II in renal fibrosis" HYPERTENSION,XX,XX, vol. 31, no. 1, PART 02, January 1998 (1998-01), pages 181-188, XP002103527 ISSN: 0194-911X the whole document	1-51
X,Y	PETERS ET AL: "Therapeutic reduction of TGF -beta and matrix proteins in Thy 1-induced glomerulonephritis is enhanced by angiotensin blockade at higher doses and further enhanced by addition of low protein diet" JOURNAL OF THE AMERICAN SOCIETY OF NEPHROLOGY,US,WILLIAMS AND WILKINS, BALTIMORE, MD, vol. 8, September 1997 (1997-09), page 524A XP002103518 ISSN: 1046-6673 the whole document	1-51
X,Y	BORDER W A ET AL: "Evidence that TGF - beta should be a therapeutic target in diabetic nephropathy 'editorial;comment!" KIDNEY INTERNATIONAL,US,NEW YORK, NY, vol. 54, no. 4, October 1998 (1998-10), pages 1390-1391, XP002103519 ISSN: 0085-2538 the whole document	1-51

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X,Y	ISAKA Y ET AL: "DECORIN GENE THERAPY FOR EXPERIMENTAL GLOMERULONEPHRITIS" JOURNAL OF THE AMERICAN SOCIETY OF NEPHROLOGY,US,WILLIAMS AND WILKINS, BALTIMORE, MD, vol. 6, no. 3, 1 September 1995 (1995-09-01), page 897 XP002005516 ISSN: 1046-6673 the whole document	1-51
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X,Y	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; February 1999 (1999-02) ISAKA YOSHITAKA ET AL: "Gene therapy by transforming growth factor-beta receptor-IgG Fc chimera suppressed extracellular matrix accumulation in experimental glomerulonephritis." Database accession no. PREV199900125784 XP002152362 abstract & KIDNEY INTERNATIONAL, vol. 55, no. 2, February 1999 (1999-02), pages 465-475, ISSN: 0085-2538	1-51

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,Y	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1998 PETERS H ET AL: "Angiotensin II blockade reduces glomerular transforming growth factor beta and matrix protein synthesis in undiseased rats." Database accession no. PREV199900120025 XP002152363 abstract & KIDNEY & BLOOD PRESSURE RESEARCH, vol. 21, no. 2-4, 1998, pages 131-132, Congress of Nephrology 1998 Joint Scientific Meeting of the Society Nephrology;Erlangen, Germany; September 19-22, 1998 ISSN: 1420-4096 ---	1-51
X,Y	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1998 HARAGUCHI MASASHI ET AL: "Recombinant tissue type plasminogen activator (rt-PA) promotes glomerular plasmin generation and extracellular matrix (ECM) turnover in anti-Thy-1 nephritis." Database accession no. PREV199900009520 XP002152364 abstract & JOURNAL OF THE AMERICAN SOCIETY OF NEPHROLOGY, vol. 9, no. PROGRAM AND ABSTR. ISSUE, 1998, page 517A 31st Annual Meeting of the American Society of Nephrology;Philadelphia, Pennsylvania, USA; October 25-28, 1998 ISSN: 1046-6673 ---	1-51
X,Y	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; January 1998 (1998-01) BORDER WAYNE A ET AL: "Interactions of transforming growth factor-beta and angiotensin II in renal fibrosis." Database accession no. PREV199800129278 XP002152365 abstract & HYPERTENSION (DALLAS), vol. 31, no. 1 PART 2, January 1998 (1998-01), pages 181-188, ISSN: 0194-911X --- -/--	1-51

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X,Y	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1996 ISAKA YOSHITAKA ET AL: "Gene therapy by skeletal muscle expression on decorin prevents fibrotic disease in rat kidney." Database accession no. PREV199698793292 XP002152366 abstract & NATURE MEDICINE, vol. 2, no. 4, 1996, pages 418-423, ISSN: 1078-8956 -----	1-51

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

In view of the large number of independent claims directed to not necessarily related subject-matter this application does not meet the requitrements of Art. 65 PCT. The search had to be limited to what appears to be the general inventive idea (i.e., the use of compounds inhibiting TGF beta and/or reducing extracellular matrix for treating fibrotic diseases), and, more specifically, to the use of the specific compounds mentioned in the pharmacological examples.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern. nal Application No
PCT/US 00/00179

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9934823 A	15-07-1999	AU 1826799 A	26-07-1999
WO 9625178 A	22-08-1996	US 5824655 A	20-10-1998
		AU 694621 B	23-07-1998
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		JP 7504650 T	25-05-1995
		NO 942072 A	03-08-1994

TENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
30434.4WO01		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US 00/00179	05/01/2000	05/01/1999
Applicant		
UNIVERSITY OF UTAH et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 8 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

In view of the large number of independent claims directed to not necessarily related subject-matter this application does not meet the requitrements of Art. 65 PCT. The search had to be limited to what appears to be the general inventive idea (i.e., the use of compounds inhibiting TGF beta and/or reducing extracellular matrix for treating fibrotic diseases), and, more specifically, to the use of the specific compounds mentioned in the pharmacological examples.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/00179

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/00 A61P13/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X,Y	WO 93 09800 A (UNIV UTAH ;UNIV CALIFORNIA (US); JOLLA CANCER RES FOUND (US)) 27 May 1993 (1993-05-27) the whole document	1-51

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[X] Further documents are listed in the continuation of box C.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
8 November 2000	28/11/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Orviz Diaz, P

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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X,Y	BORDER W ET AL: "Interactions of transforming growth factor-beta and angiotensin II in renal fibrosis" HYPERTENSION,XX,XX, vol. 31, no. 1, PART 02, January 1998 (1998-01), pages 181-188, XP002103527 ISSN: 0194-911X the whole document	1-51
X,Y	PETERS ET AL: "Therapeutic reduction of TGF -beta and matrix proteins in Thy 1-induced glomerulonephritis is enhanced by angiotensin blockade at higher doses and further enhanced by addition of low protein diet" JOURNAL OF THE AMERICAN SOCIETY OF NEPHROLOGY,US,WILLIAMS AND WILKINS, BALTIMORE, MD, vol. 8, September 1997 (1997-09), page 524A XP002103518 ISSN: 1046-6673 the whole document	1-51
X,Y	BORDER W A ET AL: "Evidence that TGF - beta should be a therapeutic target in diabetic nephropathy 'editorial;comment!" KIDNEY INTERNATIONAL,US,NEW YORK, NY, vol. 54, no. 4, October 1998 (1998-10), pages 1390-1391, XP002103519 ISSN: 0085-2538 the whole document	1-51

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X,Y	ISAKA Y ET AL: "DECORIN GENE THERAPY FOR EXPERIMENTAL GLOMERULONEPHRITIS" JOURNAL OF THE AMERICAN SOCIETY OF NEPHROLOGY,US,WILLIAMS AND WILKINS, BALTIMORE, MD, vol. 6, no. 3, 1 September 1995 (1995-09-01), page 897 XP002005516 ISSN: 1046-6673 the whole document ---	1-51
X,Y	BORDER W A ET AL: "SUPPRESSION OF EXPERIMENTAL GLOMERULONEPHRITIS BY ANTISERUM AGAINSTTRANSFORMING GROWTH FACTOR BETA1" NATURE, 26 July 1990 (1990-07-26), XP002025313 the whole document ---	1-51
X,Y	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; November 1998 (1998-11) PETERS HARM ET AL: "Targeting TGF-beta overexpression in renal disease: Maximizing the antifibrotic action of angiotensin II blockade." Database accession no. PREV199800515448 XP002152361 abstract & KIDNEY INTERNATIONAL, vol. 54, no. 5, November 1998 (1998-11), pages 1570-1580, ISSN: 0085-2538 ---	1-51
X,Y	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; February 1999 (1999-02) ISAKA YOSHITAKA ET AL: "Gene therapy by transforming growth factor-beta receptor-IgG Fc chimera suppressed extracellular matrix accumulation in experimental glomerulonephritis." Database accession no. PREV199900125784 XP002152362 abstract & KIDNEY INTERNATIONAL, vol. 55, no. 2, February 1999 (1999-02), pages 465-475, ISSN: 0085-2538 --- -/--	1-51

1

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X,Y	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1998 PETERS H ET AL: "Angiotensin II blockade reduces glomerular transforming growth factor beta and matrix protein synthesis in undiseased rats." Database accession no. PREV199900120025 XP002152363 abstract & KIDNEY & BLOOD PRESSURE RESEARCH, vol. 21, no. 2-4, 1998, pages 131-132, Congress of Nephrology 1998 Joint Scientific Meeting of the Society Nephrology;Erlangen, Germany; September 19-22, 1998 ISSN: 1420-4096 ---	1-51
X,Y	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1998 HARAGUCHI MASASHI ET AL: "Recombinant tissue type plasminogen activator (rt-PA) promotes glomerular plasmin generation and extracellular matrix (ECM) turnover in anti-Thy-1 nephritis." Database accession no. PREV199900009520 XP002152364 abstract & JOURNAL OF THE AMERICAN SOCIETY OF NEPHROLOGY, vol. 9, no. PROGRAM AND ABSTR. ISSUE, 1998, page 517A 31st Annual Meeting of the American Society of Nephrology;Philadelphia, Pennsylvania, USA; October 25-28, 1998 ISSN: 1046-6673 ---	1-51
X,Y	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; January 1998 (1998-01) BORDER WAYNE A ET AL: "Interactions of transforming growth factor-beta and angiotensin II in renal fibrosis." Database accession no. PREV199800129278 XP002152365 abstract & HYPERTENSION (DALLAS), vol. 31, no. 1 PART 2, January 1998 (1998-01), pages 181-188, ISSN: 0194-911X --- -/--	1-51

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X,Y	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1996 ISAKA YOSHITAKA ET AL: "Gene therapy by skeletal muscle expression on decorin prevents fibrotic disease in rat kidney." Database accession no. PREV199698793292 XP002152366 abstract & NATURE MEDICINE, vol. 2, no. 4, 1996, pages 418-423, ISSN: 1078-8956 -----	1-51

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 00/00179

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
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			NO	941821 A	14-07-1994
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			US	5705609 A	06-01-1998
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			AU	670770 B	01-08-1996
			AU	3242993 A	28-06-1993
			AU	7051896 A	16-01-1997
			CA	2124591 A	10-06-1993
			DE	69228700 D	22-04-1999
			DE	69228700 T	09-09-1999
			DK	616536 T	11-10-1999
			EP	0616536 A	28-09-1994
			FI	942622 A	27-07-1994
			JP	7504650 T	25-05-1995
			NO	942072 A	03-08-1994

XP-002152361

(C) BIOSIS / BIOSIS

AN - PREV199800515448

TI - Targeting TGF-beta overexpression in renal disease: Maximizing the antifibrotic action of angiotensin II blockade.

IW - ** Major Concepts **
Pharmacology; Urinary System (Chemical Coordination and Homeostasis)
** Diseases **
glomerular fibrosis: urologic disease; glomerulonephritis: urologic disease
** Organisms **
rat (Muridae)
** Taxanotes **
Animals; Chordates; Mammals; Nonhuman Mammals; Nonhuman Vertebrates; Rodents; Vertebrates
** Super Taxa **
Muridae: Rodentia, Mammalia, Vertebrata, Chordata, Animalia
** Chemicals and Biochemicals **
enalapril: angiotensin-converting enzyme inhibitor-drug, pharmacodynamics, renal-acting-drug; losartan: angiotensin II blockade, renal-acting-drug, pharmacodynamics, antifibrotic action, angiotensin II receptor blocker; transforming growth factor-beta: renal disease overexpression

PD 11.1998
P: 1-2 (2)

AU - Peters Harm; Border Wayne A; Noble Nancy A
AUAF- Div. Nephrol., Univ. Utah Sch. Med., Salt Lake City, UT 84132;
- USA

PUB - Kidney International
- Nov., 1998
VOL - 54
NR - 5
PG - 1570-1580
PD - 1998-11-00
DT - Article
LA - English (EN)
IRN - ISSN 0085-2538

AB - Background. Overproduction of transforming growth factor-beta (TGF-beta) is a key mediator of extracellular matrix accumulation in fibrotic diseases. We hypothesized that the degree of reduction of pathological TGF-beta expression can be used as a novel index of the antifibrotic potential of angiotensin II (Ang II) blockade in renal disease. Methods. One day after induction of Thy 1.1 glomerulonephritis, rats were treated with increasing doses of the Ang I converting enzyme (ACE) inhibitor enalapril and/or the Ang II receptor blocker losartan in the drinking water. Six days after disease induction the therapeutic effect on glomerular TGF-beta overexpression was evaluated. Results. Both enalapril and losartan reduced TGF-beta overproduction in a dose-dependent manner, showing a moderate reduction at doses known to control blood pressure in renal forms of hypertension. A maximal reduction in TGF-beta expression of approximately 45% was seen for both drugs starting at 100 mg/liter enalapril and 500 mg/liter losartan, with no further reduction at doses of enalapril up to 1000 mg/liter or losartan up to 2500 mg/liter. Co-treatment with both drugs was not superior to single therapy. Consistent with our hypothesis that reduction in TGF-beta expression is a valid target, other disease measures, including glomerular matrix accumulation, glomerular production and mRNA expression of the matrix protein fibronectin and the protease inhibitor plasminogen-activator-inhibitor type 1 (PAI-1) closely followed TGF-beta expression. Conclusions. The data suggest that these therapies act through very similar pathways and that, in order to more effectively treat renal fibrosis, these drugs must be combined with

other drugs that t by different mechanism.
RN - 11128-99-7 ANGIOTENSIN II; 75847-73-3 ENALAPRIL; 9015-82-1
ANGIOTENSIN-CONVERTING ENZYME; 114798-26-4 LOSARTAN
PBC - 86375
PCC - 22002*12512-14501-15501-

XP-002152362

AN - PREV199800129278
TI - Interactions of transforming growth factor-beta and angiotensin II in renal fibrosis.
P.D: 01.1998 P. 1-2 (2)
IW - ** Major Concepts **
Biochemistry and Molecular Biophysics; Urinary System (Chemical Coordination and Homeostasis)
** Diseases **
acute renal failure: urologic disease; renal fibrosis: prevention, urologic disease
** Parts, Structures, Systems of Organisms **
glomerulus: excretory system, hemodynamic alteration; juxtaglomerular cells: excretory system; kidney: excretory system
** Organisms **
animal (Animalia); human (Hominidae)
** Taxanotes **
Animals; Chordates; Humans; Mammals; Primates; Vertebrates
** Super Taxa **
Animalia; Hominidae: Primates, Mammalia, Vertebrata, Chordata, Animali
** Chemicals and Biochemicals **
angiotensin II: interaction; transforming growth factor-beta: cellular release, interaction, matrix accumulation, therapeutic implications
AW - ** Miscellaneous Descriptors **
renin-angiotensin system components, inhibition
AU - Border Wayne A; Noble Nancy A
AUAF- Div. Nephrol. Hypertension, Dep. Med., Univ. Utah Health Sci. Cent., 50 N. Medical Dr., Salt Lake City, UT 84132;
- USA
PUB - Hypertension (Dallas)
- Jan., 1998
VOL - 31
NR - 1 PART 2
PG - 181-188
PD - 1998-01-00
DT - Literature Review
LA - English (EN)
IRN - ISSN 0194-911X
AB - Overproduction of transforming growth factor-beta clearly underlies tissue fibrosis in numerous experimental and human diseases. Transforming growth factor-beta's powerful fibrogenic action results from simultaneous stimulation of matrix protein synthesis, inhibition of matrix degradation, and enhanced integrin expression that facilitates matrix assembly. In animals, overexpression of transforming growth factor-beta by intravenous injection, transient gene transfer, or transgene insertion has shown that the kidney is highly susceptible to rapid fibrosis. The same seems true in human disease, where excessive transforming growth factor-beta has been demonstrated in glomerulonephritis, diabetic nephropathy, and hypertensive glomerular injury. A possible explanation for the kidney's particular susceptibility to fibrosis may be the recent discovery of biologically complex interactions between the renin-angiotensin system and transforming growth factor-beta. Alterations in glomerular hemodynamics can activate both the renin-angiotensin system and transforming growth factor-beta Components of the renin-angiotensin system act to further stimulate production of transforming growth factor-beta and plasminogen activator inhibitor leading to rapid matrix accumulation. In volume depletion, transforming growth factor-beta is released from juxtaglomerular cells and may act synergistically with angiotensin II to accentuate vasoconstriction and acute renal failure. Interaction of

the renin-angiot in system and transformi growth factor-beta has important clinical implications. The protective effect of inhibition of the renin-angiotensin system in experimental and human kidney diseases correlates closely with the suppression of transforming growth factor-beta production. This suggests that transforming growth factor-beta, in addition to blood pressure, should be a therapeutic target. Higher doses or different combinations of drugs that block the renin-angiotensin system or entirely new drug strategies may be needed to achieve a greater antifibrotic effect.

RN - 11128-99-7 ANGIOTENSIN II
PBC - 33000 86215
PCC - 15501*02502-02506-02508-10060-12512-14501-17002-

AN - PREV199900009520
TI - Recombinant tissue type plasminogen activator (rt-PA) promotes glomerular plasmin generation and extracellular matrix (ECM) turnover in anti-Thy-1 nephritis.
IW - ** Major Concepts **
Cell Biology; Immune System (Chemical Coordination and Homeostasis); Urinary System (Chemical Coordination and Homeostasis)
** Diseases **
anti-Thy-1 nephritis: immune system disease, urologic disease
** Organisms **
rat (Muridae)
** Taxanotes **
Animals; Chordates; Mammals; Nonhuman Mammals; Nonhuman Vertebrates; Rodents; Vertebrates
** Super Taxa **
Muridae: Rodentia, Mammalia, Vertebrata, Chordata, Animalia
** Chemicals and Biochemicals **
fibrin; recombinant tissue type plasminogen activator: antifibrotic potential
AW - ** Miscellaneous Descriptors **
Meeting Abstract; Meeting Poster
AU - Haraguchi Masashi; Border Wayne A; Noble Nancy A
AUAF- Div. Nephrol., Univ. Utah Sch. Med., Salt Lake City, UT;
- USA
PUB - Journal of the American Society of Nephrology
- Sept., 1998
VOL - 9
NR - PROGRAM AND ABSTR. ISSUE
PG - 517A
CONF- 31st Annual Meeting of the American Society of Nephrology; Philadelphia, Pennsylvania, USA; October 25-28, 1998
PD - 1998-00-00
DT - Meeting
LA - English (EN)
IRN - ISSN 1046-6673
RN - 9001-91-6 PLASMINOGEN; 105857-23-6 RT-PA; 9001-90-5 PLASMIN
PBC - 86375
PCC - 34502*02506-10060-15001-15501-00520-

XP 002005516

P.D. 09-'95
p. 894 = 1

C07K14/47A15

1261

DECORIN GENE THERAPY FOR EXPERIMENTAL GLOMERULONEPHRITIS Y Isaka*, DK Brees, K Ikegaya*, Y Kaneda*, E Imai, NA Noble & WA Border, Division of Nephrology, University of Utah, Salt Lake City, UT & Osaka University, Suita, Japan.

Overproduction of transforming growth factor-ß1 (TGF-ß1) is implicated in the pathogenesis of many fibrotic diseases through its actions to increase extracellular matrix deposition. The small proteoglycan decorin binds to and neutralizes the activity of TGF-ß1. We have shown that injection of recombinant decorin prevents matrix deposition in glomerulonephritis induced by antithymocyte serum (ATS). However, production of decorin is expensive and delivery must be by intravascular injection. To overcome these limitations, we delivered therapeutic decorin to kidney by direct gene transfer into skeletal muscle. Initially, cultured myocytes transfected with decorin gene were found to secrete 11 times more decorin than myocytes transfected with the chloramphenicol acetyltransferase (CAT) control gene. When decorin gene was transferred directly into skeletal muscle of normal rats, increased staining for decorin was detected in liver, lung and kidney after 3 and 7 days, indicating systemic delivery of decorin to these tissues. Finally, decorin gene transferred into skeletal muscle of nephritic rats, either one day before or one day after induction of glomerulonephritis with ATS, lead to marked reductions in manifestations of disease including TGF-ß1 mRNA and TGF-ß1 protein production, glomerular matrix accumulation and proteinuria compared with CAT transfected control rats. These results show the therapeutic potential of decorin gene therapy for fibrotic diseases of the kidney and other organs associated with TGF-ß1 overproduction.

AN - PREV199698793292

TI - Gene therapy by skeletal muscle expression on decorin prevents fibrotic disease in rat kidney.

IW - ** Major Concepts **
Endocrine System (Chemical Coordination and Homeostasis); Genetics; Metabolism; Muscular System (Movement and Support); Pathology; Urinary System (Chemical Coordination and Homeostasis)
** Organisms **
rat (Muridae)
** Taxanotes **
animals; chordates; mammals; nonhuman mammals; nonhuman vertebrates; rodents; vertebrates
** Super Taxa **
Muridae: Rodentia, Mammalia, Vertebrata, Chordata, Animalia

AW - ** Miscellaneous Descriptors **
EXTRACELLULAR MATRIX ACCUMULATION; GLOMERULONEPHRITIS; PROTEINURIA; TRANSFORMING GROWTH FACTOR-BETA 1

AU - Isaka Yoshitaka; Brees Douglas K; Ikegaya Kazuko; Kaneda Yasufumi; Imai Enyu; Noble Nancy A; Border Wayne A

AUAF- Div. Nephrol., Univ. Utah Sch. Med., Salt Lake City, UT 84132;
- USA

PUB - Nature Medicine
- 1996
VOL - 2
NR - 4
PG - 418-423
PD - 1996-00-00
DT - Article
LA - English (EN)
IRN - ISSN 1078-8956

AB - There are currently no effective therapies for progressive fibrotic diseases. Recent evidence has implicated overproduction of transforming growth factor-beta-1 (TGF-beta-1) as a major cause of tissue fibrosis. Furthermore, this evidence implies that inhibitors of TGF-beta-1 may be clinically useful as antifibrotic agents. The proteoglycan decorin is a known inhibitor of TGF-beta-1. In a rat model of glomerulonephritis we have shown that fibrosis is mediated by TGF-beta-1. We report here that transfer of decorin cDNA into rat skeletal muscle increases the amount of decorin messenger RNA and protein present in skeletal muscle and decorin protein present in kidney, where it has a marked therapeutic effect on fibrosis induced by glomerulonephritis. Transfected glomerulonephritic rats showed a significant reduction in levels of glomerular TGF-beta-1 mRNA and TGF-beta-1 protein, extracellular matrix accumulation and proteinuria. These results demonstrate the potential of gene therapy as a novel treatment for fibrotic diseases caused by TGF-beta-1.

PBC - 86375
PCC - 03508-10064 12508-13012-13020-15506-17002-17506*

(C) BIOSIS / BIOSIS XP-0021523

AN - PREV199900125784
TI - Gene therapy by transforming growth factor-beta receptor-IgG Fc chimera suppressed extracellular matrix accumulation in experimental glomerulonephritis.
IW - ** Major Concepts **
Genetics; Immune System (Chemical Coordination and Homeostasis); Urinary System (Chemical Coordination and Homeostasis)
** Diseases **
glomerulonephritis: experimental disease model, urologic disease; glomerulosclerosis
** Chemicals and Biochemicals **
transforming growth factor-beta; transforming growth factor-beta receptor-immunoglobulin G Fc chimera
AW - ** Alternate indexing **
Glomerulonephritis (MeSH); Glomerulosclerosis, Focal (MeSH)
- ** Miscellaneous Descriptors **
gene therapy
AU - Isaka Yoshitaka; Akagi Yoshitaka; Ando Yutaka; Tsujie Michiko; Sudo Tetsuo; Ohno Noriko; Border Wayne A; Noble Nancy A; Kaneda Yasufumi; Hori Masatsugu; Imai Enyu
AUAF- First Dep. Med., Osaka Univ. Sch. Med., 2-2 Yamadaoka, Suita, Osaka 565-0871;
- Japan
PUB - Kidney International
- Feb., 1999
VOL - 55
NR - 2
PG - 465-475
PD - 1999-02-00
DT - Article
LA - English (EN)
IRN - ISSN 0085-2538
AB - Background. The evidence that transforming growth factor-beta (TGF-beta) is a key mediator in the pathogenesis of fibrotic diseases is now supported by several lines of investigation. This evidence provides a certain base for targeting TGF-beta as an antifibrotic agent. Methods. We generated a chimeric cDNA, termed TGFbetaRII/Fe, encoding an extracellular domain of the TGF-beta type II receptor fused to the IgG-Fc domain, and tested whether TGFbetaRII/Fc could be a novel strategy for treating glomerular diseases. Results. In cultured BNul-7 cells, recombinant TGFbetaRII/Fc reversed the antiproliferative response induced by TGF-beta1. In addition, TGFbetaRII/Fc diminished the TGF-beta1-induced production of EIIIA-positive fibronectin in cultured normal rat kidney cells. We then introduced the chimeric cDNA into the muscle of the nephritic rats by the hemagglutinating virus of Japan liposome-mediated gene transfer method in order to delivery of chimeric molecules. Treatment with TGFbetaRII/Fc gene transfection could suppress the glomerular TGF-beta mRNA in nephritic rats with a comparable effect in the reduction of extracellular matrix accumulation. Conclusion. TGFbetaRII/Fc successfully inhibited the action of TGF-beta in vitro and in vivo, and gene therapy by chimeric TGFbetaRII/Fc might be feasible for the therapy of glomerulosclerosis.
PCC - 03506*12512-15501-34502-

AN - PREV199900120025
TI - Angiotensin II blockade reduces glomerular transforming growth factor beta and matrix protein synthesis in undiseased rats.
IW - ** Major Concepts **
Pharmacology; Urinary System (Chemical Coordination and Homeostasis)
** Organisms **
Sprague-Dawley rat (Muridae): undiseased animal model
** Taxanotes **
Animals; Chordates; Mammals; Nonhuman Mammals; Nonhuman Vertebrates; Rodents; Vertebrates
** Super Taxa **
Muridae: Rodentia, Mammalia, Vertebrata, Chordata, Animalia
** Chemicals and Biochemicals **
enalapril: angiotensin-converting enzyme inhibitor, renal-acting-drug; losartan: angiotensin II type 1 receptor blocker, renal-acting-drug; matrix protein: angiotensin II blockade-induced reduction, glomerular synthesis; transforming growth factor-beta: angiotensin II blockade-induced reduction, glomerular synthesis
AW - ** Miscellaneous Descriptors **
Meeting Abstract; Meeting Poster
AU - Peters H; Border W A; Ketteler M; Noble N A; Distler A
AUAF- Dep. Nephrol., Klin. Benjamin Franklin, Berlin;
- Germany
PUB - Kidney & Blood Pressure Research
- 1998
VOL - 21
NR - 2-4
PG - 131-132
CONF- Congress of Nephrology 1998 Joint Scientific Meeting of the Society Nephrology; Erlangen, Germany; September 19-22, 1998
PD - 1998-00-00
DT - Meeting
LA - English (EN)
IRN - ISSN 1420-4096
RN - 11128-99-7 ANGIOTENSIN II; 75847-73-3 ENALAPRIL; 9015-82-1 ANGIOTENSIN-CONVERTING ENZYME; 114798-26-4 LOSARTAN
PBC - 86375
PCC - 22032*10508-10808-13012-15504-17002-22003-22016-00520-10060-10064-28002

CS UNIV MELBOURNE, ENDOCRINOL UNIT, AUSTIN & REPATRIAT MED CTR, DEPT MED, AUSTIN CAMPUS, STUDLEY RD, HEIDELBERG, VIC 3084, AUSTRALIA (Reprint)
CYA AUSTRALIA
SO DIABETES, (MAR 1998) Vol. 47, No. 3, pp. 414-422.
Publisher: AMER DIABETES ASSOC, 1660 DUKE ST, ALEXANDRIA, VA 22314.
ISSN: 0012-1797.
DT Article; Journal
FS LIFE; CLIN
LA English
REC Reference Count: 51
ABSTRACT IS AVAILABLE IN THE ALL AND IALL FORMATS

NO 5/20

4/4/04 486

Sean McGarr
1635 5/20
09869820

L6 ANSWER 26 OF 40 MEDLINE DUPLICATE 10
AN 1998184615 MEDLINE
DN 98184615 PubMed ID: 9525702
TI Link between angiotensin II and TGF-beta in the kidney.
AU Wolf G
CS Department of Medicine, University of Hamburg, Germany..
wolf@uke.uni-hamburg.de
SO MINERAL AND ELECTROLYTE METABOLISM, (1998) 24 (2-3) 174-80. Ref: 56
Journal code: 7802196. ISSN: 0378-0392.
CY Switzerland
DT Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
(REVIEW, TUTORIAL)
LA English
FS Priority Journals
EM 199805
ED Entered STN: 19980514
Last Updated on STN: 19980514
Entered Medline: 19980501

L6 ANSWER 35 OF 40 MEDLINE DUPLICATE 14
AN 96163237 MEDLINE
DN 96163237 PubMed ID: 8587237
TI ACE inhibition reduces proteinuria, glomerular lesions and extracellular matrix production in a normotensive rat model of immune complex nephritis.
AU Ruiz-Ortega M; Gonzalez S; Seron D; Condom E; Bustos C; Largo R; Gonzalez E; Ortiz A; Egido J
CS Renal Unit, Fundacion Jimenez Diaz, Universidad Autonoma, Madrid, Spain.
SO KIDNEY INTERNATIONAL, (1995 Dec) 48 (6) 1778-91.
Journal code: 0323470. ISSN: 0085-2538.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 199603
ED Entered STN: 19960404
Last Updated on STN: 19960404
Entered Medline: 19960327

10605826

ANSWER 36 OF 37 CAPLUS COPYRIGHT 2003 ACS
AN 1991:507031 CAPLUS
DN 115:107031
TI Transforming growth factor-.beta.1 up-regulates type IV collagenase expression in cultured human keratinocytes
AU Salo, Tuula; Lyons, J. Guy; Rahemtulla, Firoz; Birkedal-Hansen, Henning; Larjava, Hannu
CS Dep. Oral Surg., Univ. Oulu, Oulu, SF-90220, Finland
SO Journal of Biological Chemistry (1991), 266(18), 11436-41
CODEN: JBCHA3; ISSN: 0021-9258
DT Journal
LA English

L9 ANSWER 31 OF 37 BIOTECHNO COPYRIGHT 2003 Elsevier Science B.V.
AN 1993:23273871 BIOTECHNO
TI Interleukin-1.beta. and transforming growth factor-.alpha./epidermal

Mineral and Electrolyte Metabolism

Miner Electrolyte Metab 1998;24:174–180

Gunter Wolf

Department of Medicine, Division of Nephrology and Osteology, University of Hamburg, Germany

Link between Angiotensin II and TGF-β in the Kidney

Key Words
Glomerulosclerosis
Tubulointerstitial fibrosis
Hypertrophy
Progression of renal disease

Abstract
Glomerulosclerosis and tubulointerstitial fibrosis are common morphological correlates of many end-stage kidneys. There is ample evidence that transforming growth factor-β (TGF-β) plays a major role in these alterations by directly stimulating synthesis of many extracellular matrix components and reducing collagenase production, finally leading to renal scarring. Although many factors may induce TGF-β expression in the kidney, one very interesting aspect is the link between angiotensin II (ANG II) and TGF-β. Originating from observations in vascular smooth muscle cells, there are now several additional studies showing that ANG II stimulates TGF-β expression in the kidney. Although cell culture studies have convincingly demonstrated that the vasoactive peptide directly stimulates transcription as well as bioactivation of TGF-β, the in vivo evidence is more indirect. Nevertheless, there are several pathophysiological situations including unilateral ureteral obstruction, chronic cyclosporin A nephrotoxicity, various models of hypertension, and probably diabetic nephropathy in which ANG II-mediated TGF-β induction has been demonstrated to play an important role in the progression of the disease. The fascinating aspect of this relationship between ANG II and TGF-β is the fact that hemodynamic changes as well as structural changes are linked together generating a unifying model of progression of chronic renal failure with ANG II as the key player. Angiotensin-converting enzyme (ACE) inhibitor and the more recently introduced AT_1-receptor blocker may be potential drugs to interfere with this ANG II-mediated TGF-β expression. Therefore, these drugs should not only be considered as antihypertensive medications, but should rather be viewed as renoprotective substances influencing renal remodeling by preventing local TGF-β expression.

Introduction

Many chronic renal diseases, irrespective of the primary etiology, may eventually progress to end-stage renal disease with an irreversible loss of renal tissue. Glomerulosclerosis and fibrosis of the tubulointerstitial microenvironment constitute the major morphological correlates of such end-stage kidneys. Deposition of extracellular matrix proteins including fibronectin, collagen types I, III, and IV is an important component of the scarring observed during the evolution of glomerulosclerosis and tubulointerstitial fibrosis [1]. An increase in the synthesis

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http://BioMedNet.com/karger

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and/or a decrease in turnover of these proteins is, as a simplification, responsible for the net accumulation of extracellular matrix. Although many diverse factors such as growth processes, influx of immunocompetent cells, acute or chronic injury, ischemia as well as physical stretch may all modulate the synthesis and metabolism of extracellular matrix components, it is reasonable to assume that many of these stimuli are actually mediated by the autocrine or paracrine release of growth factors and cytokines [1]. Transforming growth factor-β (TGF-β) may serve as a paradigm for a profibrogenic cytokine [2]. As reviewed in detail elsewhere in this issue, TGF-β directly stimulates transcription of many extracellular matrix genes in renal cells including mesangial, endothelial and tubular cells. On the other hand, TGF-β reduces collagenase production and simultaneously stimulates expression of tissue inhibitor of metalloproteinases (TIMP) resulting in an overall inhibition of extracellular matrix turnover [1–3]. An early feature of chronic renal disease, before glomerulosclerosis and tubulointerstitial fibrosis have yet developed, is the recruitment of macrophages/monocytes from the circulation into the local tissue [4]. Along this line, it has been reported that TGF-β may function as a chemoattractant for macrophages/monocytes under certain conditions [3]. Moreover, compensatory growth processes such as hypertrophy and/or hyperplasia are characteristic findings of chronic kidney disease because nephrons surviving the injury may undergo adaptive growth to compensate for the loss of functional renal tissue [4]. Cell culture studies have revealed that TGF-β arrests renal cells in the G_1 phase of the cell cycle and stimulates cellular hypertrophy by a variety of mechanisms including inhibition of cyclin-dependent kinases (cdk), induction of cdk inhibitors such as $p27^{Kip1}$ and interference with the phosphorylation pattern of the protein product of the retinoblastoma gene (pRB) [3]. However, it has been also demonstrated, at least in vitro, that TGF-β may exert proliferative actions on renal cells under specific conditions [5]. Thus, induction of TGF-β may explain many morphological alterations of chronically failing kidneys. Nevertheless, several pathophysiological alterations being typical of chronic renal disease such as modulation of glomerular hemodynamics and tubular transport as well as the manifestation of proteinuria cannot all be explained by induction of TGF-β. The landmark studies by Anderson et al. [6] more than a decade ago showing the superiority of angiotensin-converting enzyme (ACE) inhibitors in the prevention of renal disease suggest that angiotensin II (ANG II) plays a pivotal role in the pathophysiology of chronic renal disease [7]. More recently, an array of information indicates that ANG II is a renal growth factor having functions quite similar to those of TGF-β [7]. This seemingly contrasting finding has been reconciled by the observation that ANG II stimulates TGF-β expression in a variety of cells including the kidney.

ANG II-Stimulated Expression of TGF-β

The first evidence that ANG II may indeed induce expression of TGF-β stems from observations of vascular smooth muscle cell (VSMC) growth [8–10]. In a somewhat neglected paper in the field, Hahn et al. [8] provided the first evidence more than 6 years ago that ANG II stimulates TGF-β mRNA in cultured VSMC obtained from spontaneously hypertensive (SHR) and Wistar rats. These authors deserve full credit for the first description of a link between ANG II and TGF-β [8]. Two years later, Gibbons et al. [9] noticed that ANG II, in the presence of serum, promotes proliferation, but in the absence of serum induces only hypertrophy of cultured VSMCs. These investigators found that ANG II induces a severalfold increase in TGF-β transcripts. The increase occurred within 4 h after stimulation, depended on de novo protein synthesis, and appeared to be mediated by activation of protein kinase C (PKC). Moreover, in their culture system, ANG II not only stimulated the synthesis of latent TGF-β, but also promoted its conversion to the biologically active form [9]. Finally, a neutralizing anti-TGF-β antibody amplified the proliferative effects of ANG II. The authors concluded from their study that the hypertrophy of VSMC in the absence of serum is caused by the autocrine induction of TGF-β which subsequently arrests cells in the G_1 phase of the cell cycle [9]. Stouffer and Owens [10], working with VSMC cultures obtained from SHR, principally confirmed the findings of Hahn et al. [8] and reported that treatment of these cells with ANG II increased TGF-β activity in conditioned media suggesting increased conversion of latent to active TGF-β. Current findings derived from cardiac cells have revealed that ANG II also induces TGF-β in cultured heart endothelial cells [11] and cardiac fibroblasts [12]. Consequently, a framework is emerging in which ANG II-mediated induction of TGF-β plays a major role in cardiac hypertrophy and vascular remodeling.

Our group was the first to demonstrate that ANG II stimulates TGF-β synthesis in renal cells [13]. We had previously noticed that ANG II decreased proliferation, but induced cellular hypertrophy of cultured mouse proximal tubular cells (MCT) as well as $LLC\text{-}PK_1$ cells [14, 15].

This enlargement of tubular cells in the presence of ANG II was associated with stimulated transcription and synthesis of collagen type IV, but not type I [16]. Application of exogenous TGF-β mimicked the growth effects observed with ANG II in MCT cells (inhibition of DNA synthesis, stimulation of hypertrophy) [12]. MCT cells stimulated with ANG II expressed more TGF-β_1 mRNA and produced bioactive TGF-β [12]. These effects were transduced through AT_1-receptors. Moreover, neutralizing anti-TGF-β antibody, but not control IgG, abolished the ANG II-mediated inhibition of proliferation as well as the induction of cellular hypertrophy, indicating that the ANG II-induced growth effects were in fact mediated by TGF-β [12]. Since it had been previously reported that high glucose medium stimulates TGF-β1 expression in MCT cells [17], this finding could explain our observation that ANG II's effects on TGF-β1 synthesis were augmented in high glucose-containing medium [18]. In a subsequent study, we provided evidence that ANG II directly stimulates transcription of the TGF-β1 gene [19]. The murine as well as the human TGF-β1 promoter contains two putative transcriptional start sites with several SP1-binding and AP-2-like sequences and AP-1 binding motifs located in the 5'end [20, 21; for details, see paper by Roberts, this issue]. Transient transfection of different chimeric constructs into MCT cells revealed that ANG II stimulates TGF-β1 transcription from both start sites [19]. Since ANG II induces c-*fos* and c-*jun* in MCT cells, and the protein products of these two immediate early genes form a leucine zipper which can bind to AP-1 sites [21], it is possible that the ANG II-stimulated transcription of TGF-β1 is mediated through these AP-1 sites. Further studies revealed that the type IV collagen transcription and synthesis, stimulated by ANG II, is actually also mediated by TGF-β1 since neutralizing anti-TGF-β antibodies as well as phosphorothioate-modified TGF-β1 antisense oligonucleotides abolished the stimulated collagen type IV production [22].

We have previously demonstrated that permanent transfection of MCT cells with the c-*mas* oncogene [23], whose protein product encodes a serpentine receptor-like moiety coupled to G proteins without a hitherto identified ligand, converts the hypertrophic actions of ANG II into a proliferative response. The ANG II-associated signal transduction pathway in c-*mas* expressing cells was shifted from inhibition of adenylate cyclase, as demonstrated in MCT cells, to activation of PKC [19]. Interestingly, ANG II failed to stimulate induction of TGF-β1 protein in these c-*mas* transfected MCT cells indicating that the link between ANG II and TGF-β1 induction can be disrupted, most likely by modifying signal transduction pathways [19].

Since progression through the cell cycle is controlled by a series of cyclin and cdk complexes that may be inactivated by cdk inhibitors, we further studied the expression of the cdk inhibitor $p27^{Kip1}$ in LLC-PK_1 cells treated with ANG II. Compared with cells grown without the vasoactive peptide, stimulation with ANG II enhanced $p27^{Kip1}$ protein as detected by Western blotting, but not mRNA expression [24]. This $p27^{Kip1}$ induction was mediated through AT_1-receptors. Exogenous TGF-β also stimulated $p27^{Kip1}$ protein expression in LLC-PK_1 cells [24]. Further immunoprecipitation experiments revealed that $p27^{Kip1}$ preferentially associated with cdk4 in ANG II-treated cells and that the activity of this kinase was inhibited after ANG II-treatment, an effect that may be generated by increased $p27^{Kip1}$ binding to cyclin D1-cdk4 complexes. In contrast, $p27^{Kip1}$ was not associated with cyclin E-cdk2 complexes in cells grown in the presence of ANG II. Treatment of LLC-PK_1 cells with $p27^{Kip1}$ antisense, but not missense, oligonucleotides abolished the ANG II-mediated cell hypertrophy as measured by de novo protein synthesis and total protein content [24]. $p27^{Kip1}$ antisense oligonucleotides also facilitated entry into the S-phase of the cell cycle of ANG II-stimulated tubular cells. Our findings suggest that ANG II-mediated induction of $p27^{Kip1}$ appears pivotal in the hypertrophy induced by this peptide and elucidates the molecular mechanism associated with this growth response in proximal tubular cells.

Tubular cells grown in high glucose express more TGF-β [17]. A recent study, applying a cell culture approach, showed that captopril dose-dependently inhibited hypertrophy of tubular LLC-PK_1 cells grown in high ambient glucose [25]. Captopril also decreased TGF-β receptor types I and II protein expression dose-dependently without affecting TGF-β mRNA. These findings may indicate that captopril can reverse high-glucose-induced growth effects by decreasing TGF-β receptor protein expression [25]. Whether this effect is due to inhibition of the tubular renin-angiotensin system (RAS) or represents a direct effect of captopril due to its SH groups remains to be established.

ANG II-mediated TGF-β induction in the kidney is not restricted to the proximal tubule. It has been more recently described that ANG II treatment of rat mesangial cells in culture increases TGF-β as well as the matrix components biglycan, fibronectin, and collagen type I [26]. This response is associated with cellular hypertrophy and not proliferation [26]. Since several investigators have

also reported that ANG II actually causes proliferation rather than hypertrophy of mesangial cells that are derived from other species and/or from fetal tissue [27–29], it remains unclear whether ANG II-stimulated induction of TGF-β is a more general feature of mesangial cells in all species and in all states of maturation.

In vivo Evidence of ANG II-Mediated Stimulation of TGF-β in the Kidney

The first in vivo evidence of ANG II-stimulated TGF-β expression came from studies of water-deprived mice [30]. Dehydrated mice have stimulated plasma renin activity (PRA) and exhibit an increase in renin staining of the juxtaglomerular apparatus (JGA) [30]. Interestingly, immunohistochemistry revealed an increase in TGF-β2 which co-localized with renin in the JGA and interlobular arteries [30]. This increase in TGF-β2 expression was closely associated with hypertrophy of JGA. A similar relationship between TGF-β2 expression in the JGA and hypertrophy of these cells was also observed in potassium-depleted rats [31], a well-known stimulus of renin production, as well as in human tissues obtained from children with JGA and renal vascular hypertrophy [32]. On the other hand, exogenous TGF-β2 stimulates renin release in short-term culture of juxtaglomerular cells [33]. In summary, these data show a relationship between ANG II, renin synthesis and JGA hypertrophy which may be mediated by local TGF-β2 production.

Another animal model in which a link between ANG II and TGF-β has been studied is unilateral ureteral ligation. Rats with chronic unilateral ureteral obstruction develop tubulointerstitial fibrosis. In this model, TGF-β1 mRNA levels are increased in the obstructed kidney 3 days after surgery, but do not change significantly in the contralateral kidney [34]. Treatment with the ACE-inhibitor enalapril, but not with an inhibitor of thromboxane synthase, significantly blunted this increase in TGF-β1 mRNA [34]. Moreover, an AT_1-receptor antagonist was equally effective in abolishing the increase of TGF-β1 mRNA in the obstructed kidney [35]. These findings suggest that ANG II might have a role in stimulating TGF-β1 mRNA expression and, presumably, promote through this mechanism the subsequent development of tubulointerstitial fibrosis, in unilaterally obstructed kidneys.

A potential relationship between activation of the RAS and TGF-β1 has been also investigated in several models of hypertension. For example, Hamaguchi et al. [36] reported that TGF-β1 mRNA was elevated in kidneys of stroke-prone spontaneously hypertensive rats (SHRSP) compared with Wistar-Kyoto rats. Immunoreactive TGF-β1 protein in SHRSP was localized mainly in glomerular, preferentially epithelial cells [36]. Treatment of SHRSP with the ACE inhibitor cilazepril as well as with the calcium antagonist nifedipine (from age 13 to 25 weeks) both lowered systemic hypertension and prevented the increase in TGF-β1 expression [36]. Although treatment with nifedipine may interfere with renin release from the JGA, these studies indicate that hypertension per se rather than an increase in local ANG II concentration may be responsible for the induction of TGF-β1. We have recently observed similar findings in two-kidney, one-clip (2-K 1-C) hypertensive rats wherein glomerular TGF-β1 transcripts were upregulated in the contralateral kidney 21 days after surgery [37]. Treatment of these rats with either the AT_1-receptor antagonist losartan or with triple therapy (hydralazine, reserpine, hydrochlorothiazide) which does not antagonize the PRA, started 24 h after clipping, significantly reduced systolic blood pressure in hypertensive rats at day 21 after clipping. Both treatments were also equally effective in preventing the increase in glomerular TGF-β1 mRNA and protein expression in the contralateral kidney. Moreover, Shankland et al. [38] have shown that increased glomerular capillary hydrostatic pressure in uninephrectomized spontaneously hypertensive rats is associated with increased glomerular TGF-β expression, a response which was normalized by treatment with an ACE inhibitor. These results are in agreement with the hypothesis that TGF-β1 expression is regulated by the increase in systemic blood pressure and not directly by ANG II. One potential mechanism to explain this hypertension-induced TGF-β1 expression may be the observation that artificially generated glomerular hypertension of in vitro perfused isolated glomeruli stimulated TGF-β expression [39]. Along this line, it has been also reported that stretching of cultured mesangial cells induced TGF-β synthesis [39, 40]. Interestingly, this response was augmented in the presence of ANG II suggesting that the vasoactive peptide may amplify the pressure-induced TGF-β1 expression [40].

Another pathophysiological situations in which ANG II-mediated TGF-β expression may be important is chronic cyclosporin A (CSA) nephrotoxicity. Although acute CSA nephrotoxicity which is caused by renal afferent arteriolar vasoconstriction is reversible, chronic nephrotoxicity is often characterized by progressive renal failure and irreversible tubulointerstitial fibrosis. We have previously described that CSA stimulates TGF-β1 synthesis in cultured mouse proximal tubular cells as well as in

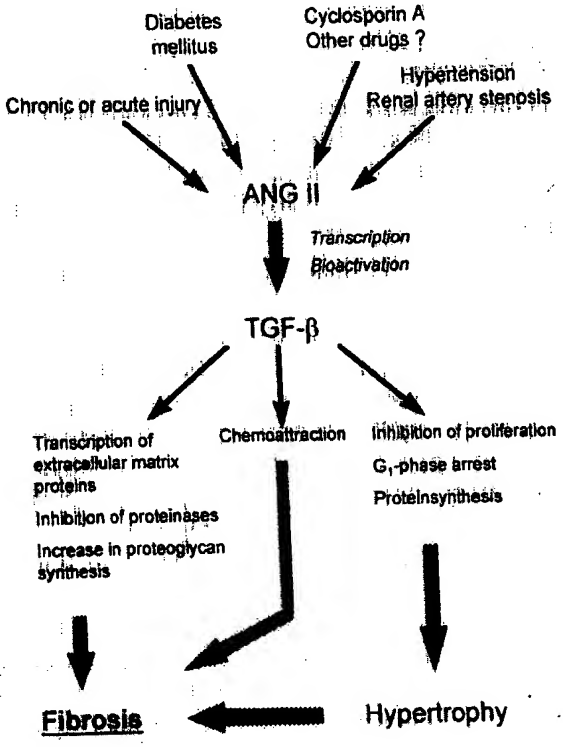

Fig. 1. Relationship between ANG II and TGF-β in the kidney. Various pathophysiological situations may induce intrarenal activation of the RAS resulting in a local increase in ANG II. After binding to its putative receptor, ANG II stimulates transcription as well as bioactivation of TGF-β in renal cells such as proximal tubular and mesangial cells. Subsequent autocrine and paracrine actions of TGF-β lead to chemoattraction of immune competent cells which may further injure the kidney, development of renal hypertrophy, and increase in extracellular matrix. These factors, in concert, lead to the development of renal fibrosis as exemplified in glomerulosclerosis and tubulointerstitial fibrosis with irreversible loss of renal function. ACE inhibitors and probably AT_1-receptor antagonists may exert part of their beneficial effects on renal function by preventing ANG II-mediated expression of TGF-β.

Table 1. Pathophysiological situations in which ANG II may stimulate TGF-β expression

In vitro observations
Vascular smooth muscle cells
Cardiac fibroblast
Heart endothelial cells
Proximal tubular cells
Mesangial cells
In vivo systems
Unilateral ureteral obstruction
Two kidneys-one clip hypertension
Chronic cyclosporin A nephrotoxicity
Water-deprivation (TGF-β2 expression in the JGA)
Diabetic nephropathy
Loss of renal tissue

vivo when the drug was injected into naive mice [41]. Subsequent reports have also demonstrated that CSA stimulates TGF-β expression in rats [42, 43]. A recent study indicates that tubulointerstitial fibrosis, induced in rats by chronic CSA application, is prevented by either an ACE inhibitor or an AT_1-receptor blocker [44]. These findings, together with our previous observations, may suggest that CSA enhances renal ANG II synthesis which, in turn, subsequently induces TGF-β1 expression leading to tubulointerstitial fibrosis. In agreement with this scenario is the observation that CSA induces recruitment of renin containing cells along the afferent arteriole leading to an activated RAS.

It has been demonstrated more than a decade ago that ACE inhibitors prevent the progression of renal disease in rats with reduced renal mass [6]. Although initially thought to be solely due to normalization of glomerular hemodynamics, subsequent studies demonstrated that not all the beneficial effects of these drugs can be contributed to hemodynamic changes [45]. Meanwhile, evidence has accumulated showing that adaptive growth processes such as compensatory hypertrophy are also very important in the progression of renal disease [46]. ANG II may be an important mediator of this compensatory renal growth after loss of nephrons [7]. In addition, it has been reported that TGF-β expression is stimulated in the remnant kidney undergoing compensatory hypertrophy [references in 4]. Consequently, it appears intriguing to conclude that some of the effects of ACE inhibitors in delaying the development of end-stage renal disease in the remnant kidney model may be due to the inhibition of TGF-β.

ACE inhibitors are currently considered firstline therapy for the management of diabetic renal disease [47]. There also exists evidence that an AT_1 blocker may have similar effects, at least in experimentally induced diabetes mellitus [48]. TGF-β is a pivotal mediator of the progressive accumulation of extracellular matrix components in the glomerular mesangium and in the tubulointerstitial space, features being typical of overt diabetic nephropathy [49, 50]. As reviewed elsewhere in this issue [see paper

by Hoffman et al., this issue] high glucose per se as well as advanced glycosylated end products stimulate TGF-β expression in renal cells. Although still controversial, there is increasing evidence that the local renal RAS may be activated in diabetes mellitus despite there is no change or even a decrease in systemic PRA [51]. Thus, an increase in local ANG II concentration may further enhance TGF-β expression in the renal microenvironment during the development of diabetic nephropathy [51].

Conclusion

Convincing evidence has accumulated over the last years showing (table 1) that ANG II stimulates TGF-β expression in the kidney. Although cell culture studies have demonstrated that the vasoactive peptide directly stimulates transcription as well as bioactivation of TGF-β, the in vivo evidence is, naturally, more indirect. Yet, there are several pathophysiological situations including unilateral ureteral obstruction, chronic CSA nephrotoxicity, various models of hypertension, and probably diabetic nephropathy, in which ANG II-mediated TGF-β induction may play an important role in the progression of the disease. Considering the fact that renal TGF-β is a key player in the development of irreversible changes such as glomerulosclerosis and tubulointerstitial fibrosis, strategies to antagonize this profibrogenic cytokine are essential to prevent the loss of functioning renal tissue (fig. 1). Despite the fact that a whole array of new approaches to interfere with TGF-β expression including application of neutralizing antibodies, antisense oligonucleotides, and overexpression of decorin have been developed [52–54], such treatments are currently limited to animal studies. However, the long-known ACE inhibitors and the more recently introduced AT_1-receptor blocker may be potential drugs to interfere with, at least ANG II-mediated, TGF-β expression. Indeed, it has been demonstrated that ACE inhibitors slow the progression of several forms of chronic renal disease in humans [55]. Thus, such drugs should not only be considered as antihypertensive medications but rather as renoprotective substances [56].

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Expression of Transforming Growth Factor-β1 and Type IV Collagen in the Renal Tubulointerstitium in Experimental Diabetes

Effects of ACE Inhibition

Richard E. Gilbert, Alison Cox, Leonard L. Wu, Terri J. Allen, U. Lennart Hulthen, George Jerums, and Mark E. Cooper

Transforming growth factor-β (TGF-β) and the renin-angiotensin system (RAS) have both been implicated in the pathogenesis of glomerulosclerosis in diabetic kidney disease. However, tubulointerstitial pathology may also be an important determinant of progressive renal dysfunction in diabetic nephropathy. In the present study, we investigated tubulointerstitial injury, TGF-β1 expression, and the effect of blocking the RAS by inhibition of ACE. We randomized 36 male SD rats to control and diabetic groups. Diabetes was induced in 24 rats by administration of streptozotocin; 12 diabetic rats were further randomized to receive the ACE inhibitor ramipril (3 mg/l drinking water). At 6 months, experimental diabetes was associated with tubulointerstitial damage, a 70% increase in expression of TGF-β1 ($P < 0.05$ vs. control), and a 120% increase in α1 (IV) collagen gene expression ($P < 0.01$ vs. control). In situ hybridization demonstrated a diffuse increase in both TGF-β1 and α1 (IV) collagen mRNA in renal tubules. In addition, intense expression of both transcripts was noted in regions of focal tubular dilatation. Administration of the ACE inhibitor ramipril prevented tubulointerstitial injury and the overexpression of TGF-β1 and α1 (IV) collagen mRNA. Changes in gene expression were accompanied by parallel changes in immunostaining for TGF-β1 and type IV collagen. The observed beneficial effects of ramipril on the tubulointerstitium in experimental diabetes suggest that this mechanism may contribute to the therapeutic effect of ACE inhibitors in diabetic nephropathy.
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Tubulointerstitial fibrosis is a typical finding in virtually all progressive renal diseases. Numerous studies have demonstrated that the magnitude of tubulointerstitial damage may be an important prognostic marker of renal outcome in many forms of primary glomerular disease (1) and that the processes of glomerular and tubulointerstitial injury are pathogenetically related (2). Recently, the molecular mechanisms that lead to the tubulointerstitial pathology in progressive renal disease have begun to be elucidated (3).

In human diabetic nephropathy, the extent of interstitial fibrosis is strongly associated with mesangial expansion, falling glomerular filtration rate (GFR), and increasing proteinuria (4–6). In experimental diabetic nephropathy, investigation has focused almost exclusively on the glomerulus, although tubulointerstitial disease also develops in this setting (7). Accumulation of extracellular matrix (ECM), first recognized as thickening of capillary basement membranes, is a characteristic pathological feature of diabetes (8) and is present in the tubulointerstitium as well as in the glomerulus (9). Intensive investigation into the pathogenesis of ECM accumulation in diabetes has consistently implicated the prosclerotic cytokine, transforming growth factor (TGF)-β, as a key mediator (10,11).

Results from recent studies of nondiabetic renal disease have suggested that the renoprotective action of blocking the renin-angiotensin system (RAS) is associated with amelioration of the overexpression of TGF-β in both the glomerulus and tubulointerstitium (12–14). These findings suggest that the efficacy of this therapeutic strategy in diabetic nephropathy (15) may also reflect changes in TGF-β synthesis. The present study was thus initiated to examine expression of TGF-β in the tubulointerstitium and the effects of blockading the RAS in experimental diabetes.

RESEARCH DESIGN AND METHODS

Animals. SD rats ($n = 36$) age 13 weeks were randomized to control and diabetic groups. Diabetes was induced in 24 rats by the intravenous administration of STZ 50 mg/kg body wt. Diabetic animals received 4 U/day long-acting insulin (Ultralente; Novo-Nordisk, Bagsvaerd, Denmark) to maintain body weight and prevent ketoacidosis. To evaluate the effects of the RAS, 12 diabetic rats were randomized to receive the ACE inhibitor ramipril (Hoechst, Frankfurt, Germany; 1 mg/kg in drinking water). The remaining 12 rats were injected with citrate buffer and served as control animals. Systolic blood pressure was measured in all animals every 4

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AER, albumin excretion rate; ECM, extracellular matrix; GFR, glomerular filtration rate; RAS, renin-angiotensin system; STZ, streptozotocin; TBM, tubular basement membrane; TGF, transforming growth factor.

weeks by tail cuff plethysmography (16). At 24 weeks' duration of diabetes, the animals were housed in metabolic cages for 24 h to obtain urine for the measurement of albumin by radioimmunoassay (17). The following day, the GFR was measured by single shot Tc^{99m}-DTPA clearance as previously described (18).

At 24 weeks' duration of diabetes, rats were anesthetized with pentobarbital sodium (Nembutal; Bomac, Asquith, Australia). The left renal artery was ligated, and the left kidney was then excised, decapsulated, and weighed. The left kidney was then bissected sagitally. The anterior half-kidney was snap frozen in liquid nitrogen and stored at –80°C for subsequent Northern blot analysis. The posterior half was then bissected once more; one-half was immersion fixed in 10% neutral buffered formalin for in situ hybridization and the other half was immersed in Methyl Carnoy's fixative for immunohistochemical studies. To reduce fixation artifacts, the right kidney was perfused in vivo at arterial pressure using an intra-aortic cannula with 0.9% saline followed by 2.5% glutaraldehyde (19). The right kidney was then excised, fixed in formalin, and embedded in paraffin for examination by light microscopy.

Because the tubulointerstitium accounts for >95% kidney volume (10), whole kidney was used as an index of tubulointerstitial gene expression by Northern blot, with more detailed structural analysis undertaken by in situ hybridization and histological studies. Blood obtained at death was collected in lithium heparin tubes, placed on ice, and immediately spun, separated, and stored at –20°C. HbA_{1c} at death was measured by high-performance liquid chromatography (20). To confirm that rats had received the appropriate drug treatment, plasma renin activity was measured by radioimmunoassay, as previously described (21). All aspects of the experiment were approved by the Animal Ethics Committee of the Austin and Repatriation Medical Centre.

Tubulointerstitial morphology. Paraffin-embedded tissue sections were stained with hematoxylin and eosin. Morphological analyses were performed with the observer blinded to the animal treatment group. The extent of injury was quantitated by a point-counting technique (22). Tubulointerstitial changes, including normal tubules, dilated tubules, inflammatory cell infiltrates, and overall interstitial spaces (cell infiltrate, fibrosis, and other spaces such as renal vessels and intertubular space) were quantitated. Under high power magnification (200×) light microscopy, five random and nonoverlapping fields from each slide were examined using a 121-point graticule to estimate the relative percentage of surface area occupied by each of the above histological parameters.

RNA preparation and analysis. Tissues were homogenized (Ultra-Turrax; Janke & Kunel, Staufen, Germany) and total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method (23). RNA purity and concentration were determined spectrophotometrically. Samples (20 µg each) were denatured and electrophoresed through 0.8% agarose formaldehyde gels. RNA integrity was verified by examination of the 28S and 18S ribosomal bands of ethidium bromide–stained material under ultraviolet light. RNA was then transferred onto nylon filters (Hybond-N, Amersham, U.K.) by capillary action and fixed by ultraviolet irradiation. Filters were hybridized with a 985-bp cDNA probe coding for rat TGF-β1 (gift of Dr. Qian, National Institutes of Health, Bethesda, MD) and a 1.8-kb pGEM 3Z cDNA probe coding for mouse α1 (IV) collagen (gift of Dr. R Timpl, Max Plank, Martinsried, Germany). The probe was labeled with [α-^{32}P]dCTP by random-primed DNA synthesis (Boehringer Mannheim, Mannheim, Germany). Hybridization was performed at 42°C for 24 h in 50% formamide, 45 mmol/l Na_2HPO_4, 5× Denhardt's solution, 0.5% SDS, and sonicated salmon sperm DNA. Filters were then washed in solutions of decreasing ionic strength and increasing temperature. Filters were then exposed to X-ray film (Kodak X-Omat; Eastman-Kodak, Rochester, NY) at –80°C for 1–5 days. The relative intensity of autoradiograms was determined by scanning densitometry (LKB Ultroscan XL, Bromma, Sweden). All results were corrected for differences in RNA loading by rehybridization with an oligonucleotide probe for 18S rRNA end-labeled with [α-^{32}P]dCTP by terminal transferase (Boehringer Mannheim).

In situ hybridization. An anti-sense riboprobe was generated as previously described (24). In brief, the cDNA coding for TGF-β1 was cloned into pBluescript KS+ (Stratagene, La Jolla, CA) and linearized with *Xba* I; an antisense riboprobe was produced using T7 RNA polymerase. The cDNA probe for α1 (IV) collagen was cloned into pGEM 3Z and linearized with *Bam*HI to produce an antisense riboprobe with SP6 RNA polymerase. Purified riboprobe length was adjusted to approximately 150 bases by alkaline hydrolysis. Sections 4 µm thick were cut onto slides precoated with 3-aminopropyltriethoxysilane and baked overnight at 37°C. Tissue sections were dewaxed and rehydrated in graded ethanol and milliQ water, equilibrated in phosphate buffer (50 mmol/l Tris-HCl, pH 7.5, 5 mmol/l EDTA), and incubated in 125 µg/ml Pronase E in phosphate buffer for 10 min at 37°C. Sections were then washed in 0.1 mol/l sodium phosphate buffer, pH 7.2, rinsed in milliQ water, dehydrated in 70% ethanol, and air dried. Hybridization buffer containing 2×10^4 cpm/µl riboprobe in 300 mmol/l NaCl, 10 mmol/l Tris-HCl, pH 7.5, 10 mmol/l Na_2HPO_4, 5 mmol/l EDTA, pH 8.0, 1× Denhardt's solution, 50% formamide, 17 mg/ml yeast RNA, and 10% wt/vol dextran sulfate was heated to 85°C for 5 min. Then 25 µl of this solution was added to each section. Hybridization was performed overnight at 60°C in 50% formamide humidified chambers. As controls for nonspecific signal, sections were incubated with sense riboprobe or treated with RNAse before hybridization. Slides were washed in 2× standard saline citrate containing 50% formamide prewarmed to 50°C to remove coverslips. Sections were then washed in the above solution for 1 h at 55°C, rinsed three more times in RNAse buffer (10 mmol/l Tris-HCl, pH 7.5, 1 mmol/l EDTA, pH 8.0, 0.5 mol/l NaCl), and then incubated with RNAse A (150 µg/ml) for 1 h at 37°C. Sections were later washed in 2× SSC for 45 min at 55°C, dehydrated in graded ethanol, air dried, and exposed to Kodak X-Omat autoradiographic film for 3 days. Slides were then dipped in Ilford K5 nuclear emulsion (Ilford, Mobberley, Cheshire, U.K.), stored in a light-free box with desiccant at 4°C for 21 days, immersed in a Kodak D19 developer, fixed in Ilford Hypan, and stained with hematoxylin and eosin.

Immunohistochemistry. Sections 4 µm thick were placed onto slides, deparaffinized, and rehydrated. Sections for type IV collagen immunohistochemistry underwent microwave oven pretreatment as previously described (25). To block endogenous peroxidase, sections were pretreated with 1% H_2O_2/methanol. In addition, sections for TGF-β immunostaining were digested with hyaluronidase (1 mg/ml in 0.1 mol/l sodium acetate) at room temperature for 30 min (13). Sections were next incubated in protein-blocking agent (Lipshaw-Immunon, Pittsburgh, PA) for 20 min at room temperature. This was followed by incubation with either polyclonal rabbit anti-human TGF-β antiserum (R & D Systems, Minneapolis, MN) or polyclonal goat anti-bovine/anti-human type IV collagen antibody (Southern Biotechnology, Birmingham, AL) for 60 min at room temperature, washed in phosphate-buffered saline, and incubated with universal biotinylated immunoglobulin (DAKO, Carpinteria, CA) and peroxidase-conjugated streptavidin (DAKO) as previously described (26). Peroxidase conjugates were subsequently localized using diaminobenzidine tetrahydrochloride as a chromogen. Sections were then counterstained with Mayer's hematoxylin and examined by two independent observers blinded to the disease status of the animal. Immunostaining was scored on a scale of 0–3, where 0 = no staining and 3 = maximum staining (adapted from Park et al. [27]). Negative controls included omitting the primary antibody or replacing it with normal rabbit IgG at an equivalent protein concentration.

Statistics. Because of a positively skewed distribution, the albumin excretion rate (AER) was logarithmically transformed before statistical analysis and expressed as the geometric mean ×/÷ tolerance factor. Other results are expressed as means ± SE unless otherwise stated. Data derived from histological and immunohistochemical studies were not normally distributed and were analyzed nonparametrically using the Kruskal-Wallis test. All other data comparing treatment groups were analyzed by analysis of variance with correction for multiple comparisons using Fisher's least significant differences test. Correlation was exam-

TABLE 1
Body weight, kidney weight, blood pressure, glycemic control, and renal function in experimental animals at 24 weeks' duration of diabetes

	Control	Diabetes	Diabetes + ramipril
n	12	12	12
Kidney weight (g)	1.9 ± 0.1	2.3 ± 0.1*	2.4 ± 0.1*
Kidney: body wt	0.29 ± 0.01	0.52 ± 0.03‡	0.51 ± 0.02‡
Mean blood pressure (mmHg)	127 ± 2	136 ± 2*	123 ± 3†
HbA_{1c} (%)	2.4 ± 0.1	4.0 ± 0.2‡	4.0 ± 0.2‡
AER (mg/day)	1.20 ×/÷ 1.2	3.96 ×/÷ 1.5‡	0.99 ×/÷ 1.3§
GFR (ml · min^{-1} · kg^{-1} body wt)	7.8 ± 0.5	11.1 ± 0.9*	9.9 ± 1.4†

*P < 0.05 vs. control; †P < 0.05 vs. diabetes; ‡P < 0.01 vs. control; §P < 0.01 vs. diabetes.

TABLE 2
Tubulointerstitial injury and immunostaining for type IV collagen and TGF-β in experimental animals at 24 weeks' duration of diabetes

	Control	Diabetes	Diabetes + ramipril
Normal tubules (%)	81 ± 2	56 ± 6‡	73 ± 5§
Dilated tubules (%)	1.7 ± 1	23 ± 6‡	8.0 ± 5†
Spaces (%)	14.3 ± 1.2	18.2 ± 1.1*	16.3 ± 1.0
Cells (%)	2.0 ± 0.3	2.5 ± 0.8	1.8 ± 0.2
Type IV collagen¶	1.5 (1–3)	3 (2–4)*	1 (0–2)†
TGF-β¶	0.5 (0–1)	1.7 (1–3)*	0.5 (0–1)†

Data are means ± SD or median (range). *P < 0.05 vs. control; †P < 0.05 vs. diabetes; ‡P < 0.01 vs. control; §P < 0.01 vs. diabetes; ¶semiquantitative scoring for immunohistochemistry.

ined by linear regression analysis. Analyses were performed using the Statview SE+ graphics package (Abacus Concepts, Calabasas, CA) on an Apple Macintosh Quadra 605 (Apple Computer, Cupertino, CA). A P value <0.05 was considered statistically significant.

RESULTS

Basic data. Rats that received STZ were all diabetic (blood glucose >15 mmol/l) with no difference in HbA_{1c} between animals treated or not treated with ramipril (Table 1). Diabetes was associated with increased kidney weight and reduced body mass when compared with control animals. These effects on body weight and kidney weight were not changed by treatment with ramipril (Table 1). Ramipril treatment was accompanied by AERs and systolic blood pressure levels similar to those of control animals and significantly less than those of untreated diabetic rats (Table 1). Diabetes was associated with increased GFRs in comparison with control and ramipril-treated diabetic animals (Table 1).

Histopathology. Kidneys of untreated diabetic rats demonstrated a variety of histopathological changes in the tubulointerstitium (summarized in Table 2). In contrast, no significant injury was noted in ramipril-treated diabetic rats.

Northern blot analysis. Compared with control animals, kidney TGF-β1 gene expression was increased by 70% (P < 0.05) and α1 (IV) collagen mRNA was increased 120% (P < 0.01) in diabetic rats compared with control animals (Figs. 1 and 2). The expression of both TGF-β1 and α1 (IV) collagen in ramipril-treated diabetic rats was reduced to levels found in control animals. No relationship was present between either TGF-β1 or α1 (IV) collagen expression and albuminuria, kidney weight, or HbA_{1c} in either diabetic group. However, a significant correlation between TGF-β1 and α1 (IV) collagen mRNA was noted in control and diabetic groups (r = 0.84, P < 0.05 and r = 0.71, P < 0.05, respectively) and approached statistical significance in ramipril-treated diabetic animals (r = 0.54, P = 0.09).

In situ hybridization. In situ hybridization of renal tissue from control animals revealed sparse hybridization for TGF-β1 and α1 (IV) collagen mRNA in the tubulointerstitium. In contrast, a diffuse increase in TGF-β1 and α1 (IV) collagen gene expression was noted throughout the nephron, particularly within proximal tubules (Figs. 3 and 4). In addition, focal areas of intense overexpression were also identified in regions of tubulointerstitial injury in kidneys of diabetic animals (Figs. 5 and 6). In these areas increased expression of TGF-β1 and α1 (IV) collagen mRNA was noted in both tubular

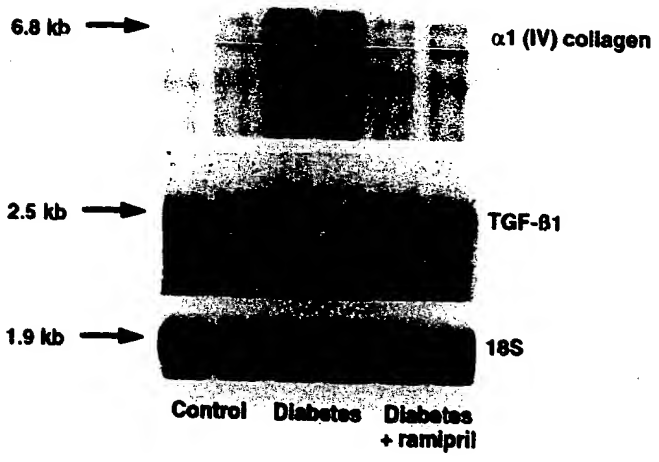

FIG. 1. Northern blot of kidney mRNA for TGF-β1 and α1 (IV) collagen in control, diabetic, and ramipril-treated diabetic rats. Increased gene expression of TGF-β1 and α1 (IV) collagen is seen in untreated diabetic animals with no significant change in 18S rRNA.

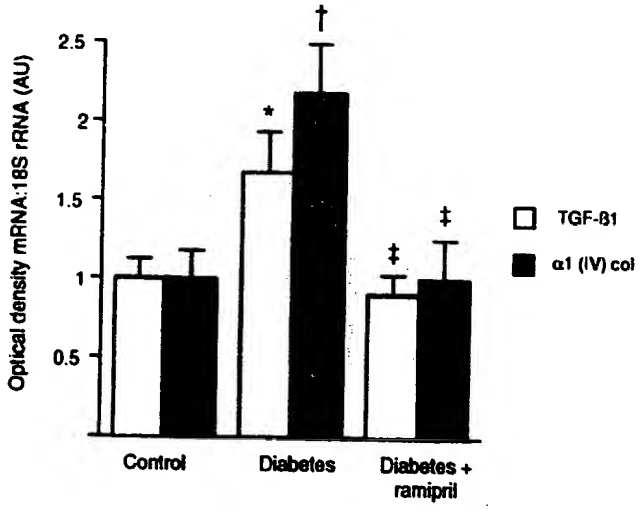

FIG. 2. Quantification of kidney TGF-β1 and α1 (IV) collagen mRNA in control, diabetic, and ramipril-treated diabetic rats by Northern blot analysis. Data are means ± SD of the ratio of optical density in arbitrary units (AUs) for mRNA to that of 18S rRNA. *P < 0.05 vs. control; †P < 0.01 vs. control; ‡P < 0.01 vs. diabetic rat.

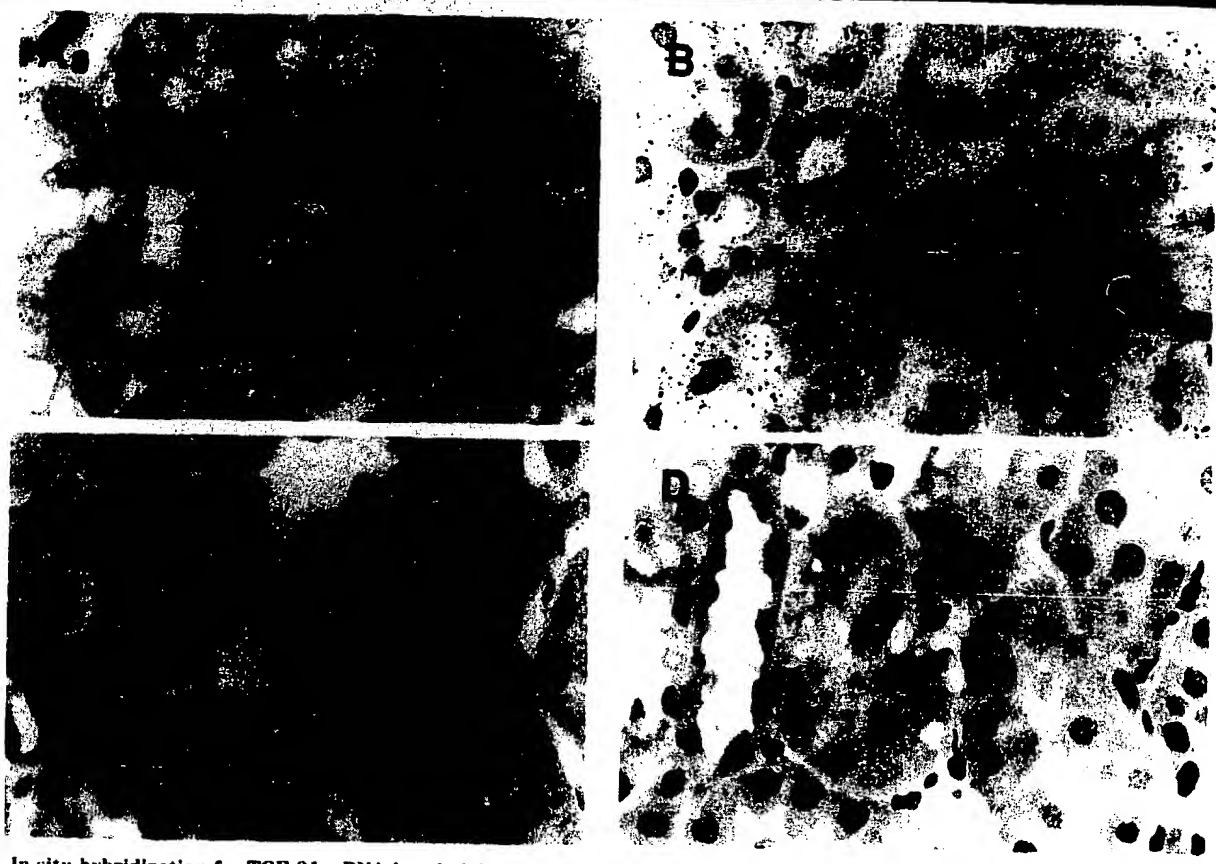

FIG. 3. In situ hybridization for TGF-β1 mRNA in tubulointerstitium from control (*A*), diabetic (*B*), and ramipril-treated diabetic (*C*) rats. Increased signal is present in kidneys from diabetic rats compared with control and ramipril-treated diabetic animals. Tissue sections incubated with sense TGF-β1 riboprobe showed absent hybridization (*D*). Original magnification ×1,000.

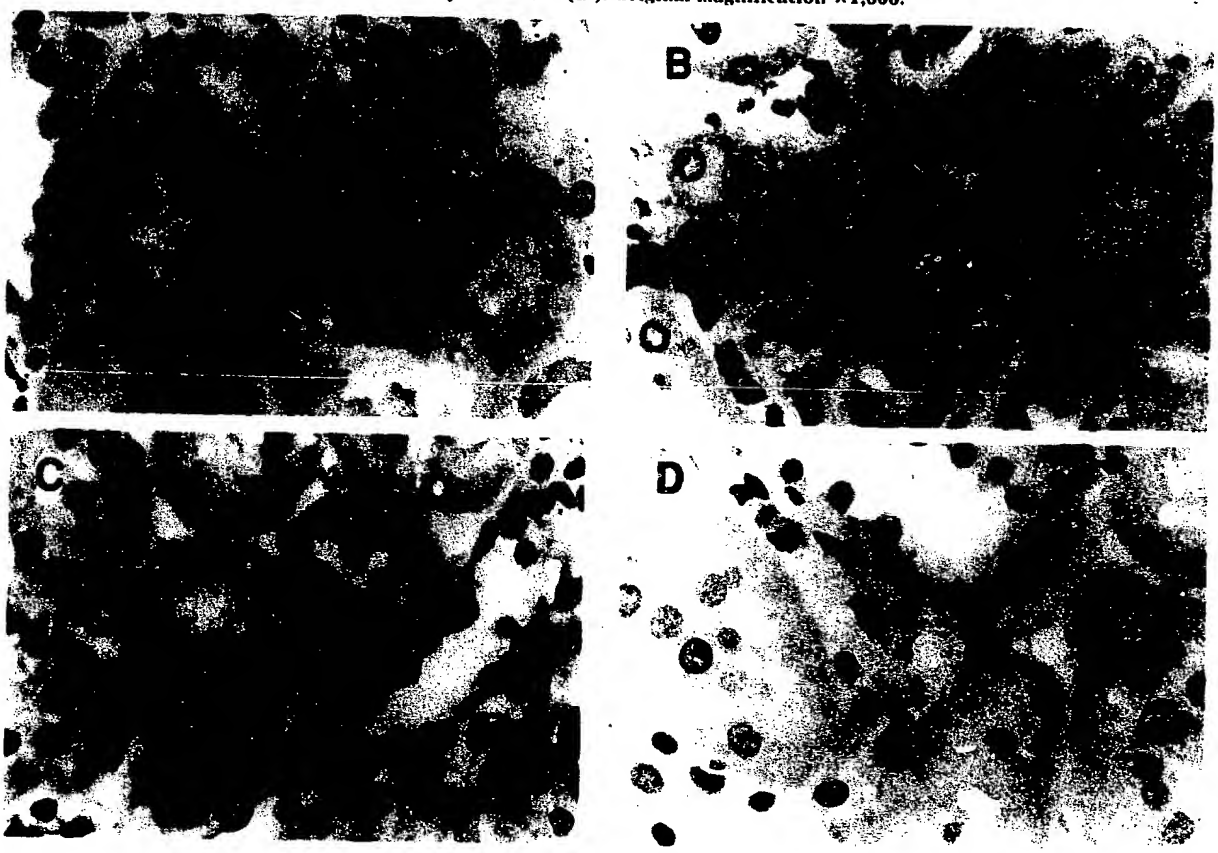

FIG. 4. In situ hybridization for α1 (IV) collagen mRNA in tubulointerstitium from control (*A*), diabetic (*B*), and ramipril-treated diabetic (*C*) rats. Increased signal is present in kidneys from diabetic rats compared with control and ramipril-treated diabetic animals. Tissue sections incubated with sense α1 (IV) collagen riboprobe showed absent hybridization (*D*). Original magnification ×1,000.

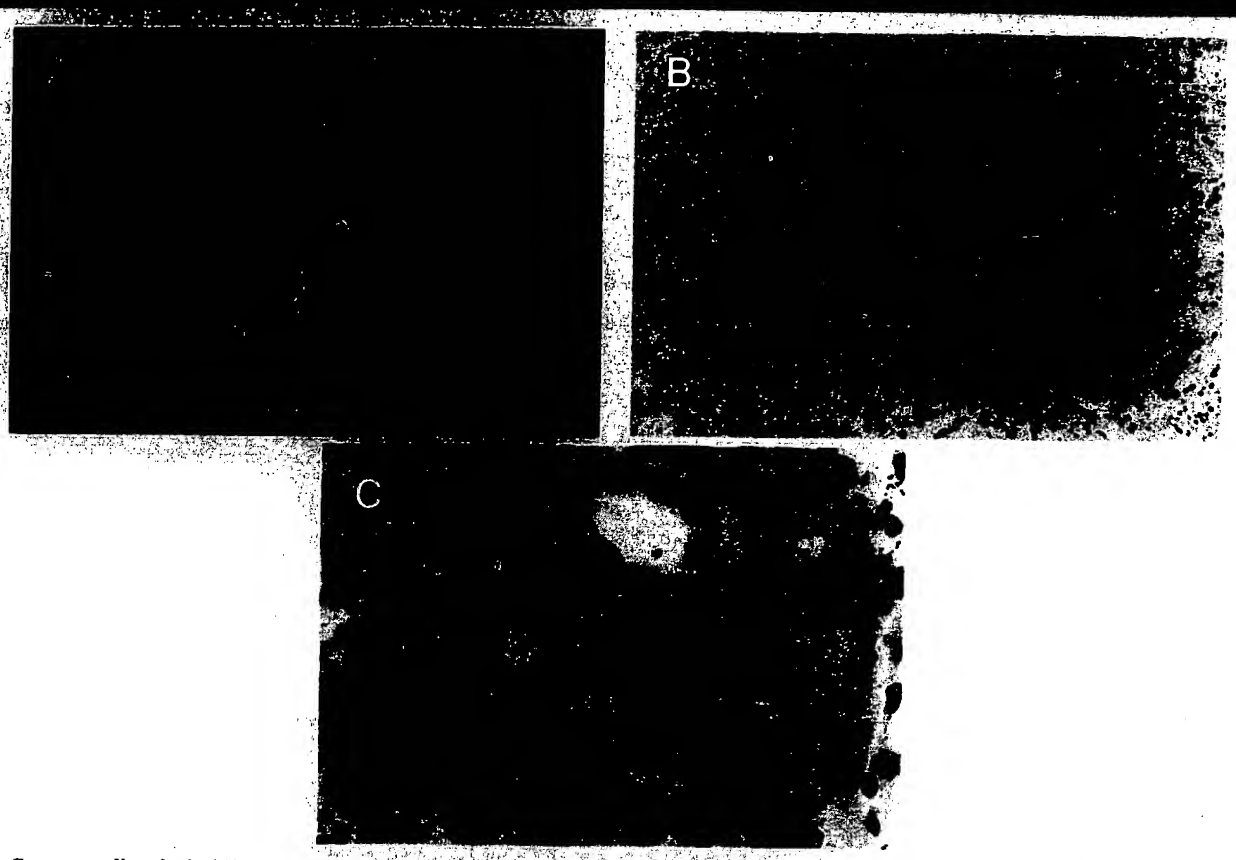

FIG. 5. Corresponding dark- (*A*) and brightfield (*B*) photomicrographs demonstrating intense expression of TGF-β1 mRNA in association with a dilated tubule from a diabetic rat. Original magnification ×200. High power view (*C*) demonstrating TGF-β1 mRNA at site of tubular pathology. Original magnification ×1,000.

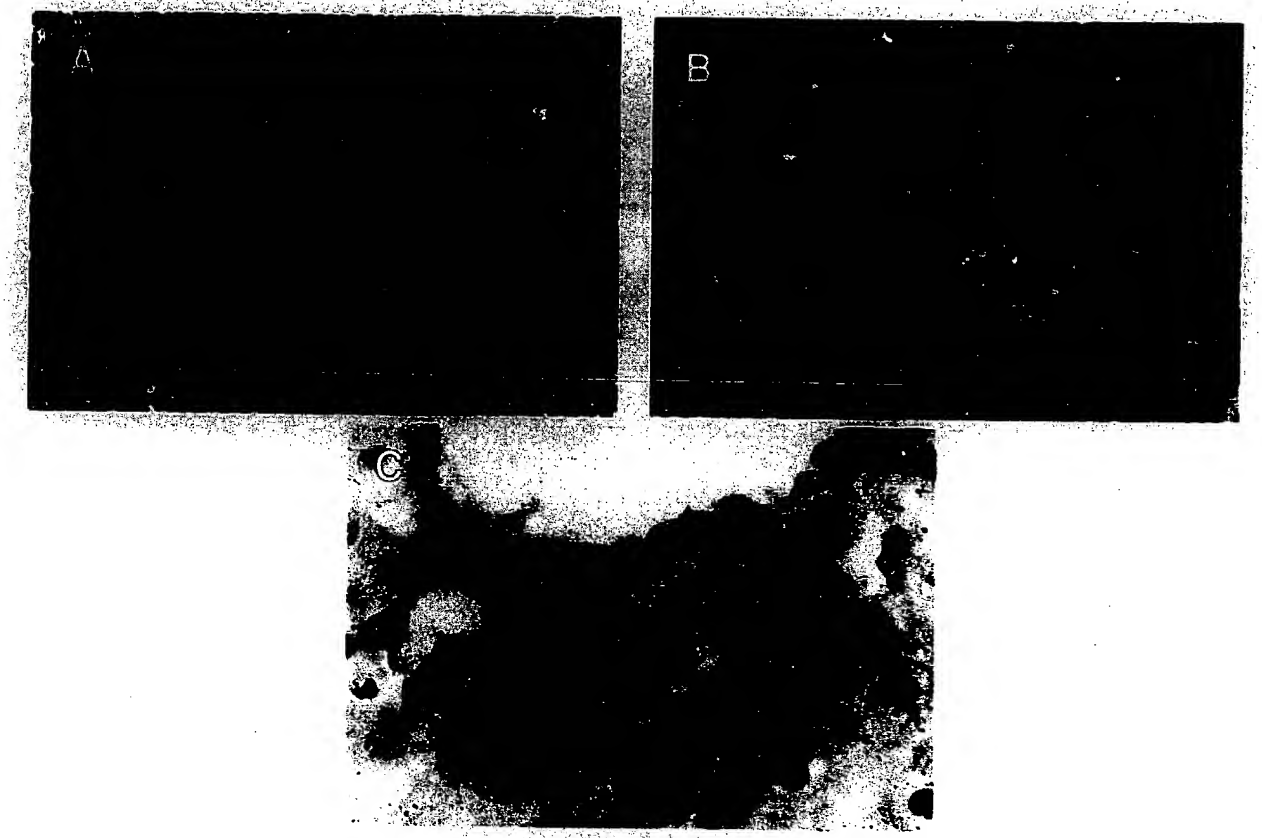

FIG. 6. Corresponding dark- (*A*) and brightfield (*B*) photomicrographs demonstrating intense expression of α1 (IV) collagen mRNA in association with a dilated tubule from a diabetic rat. Original magnification ×200. High power view (*C*) demonstrating α1 (IV) collagen mRNA at site of tubular pathology. Original magnification ×1,000.

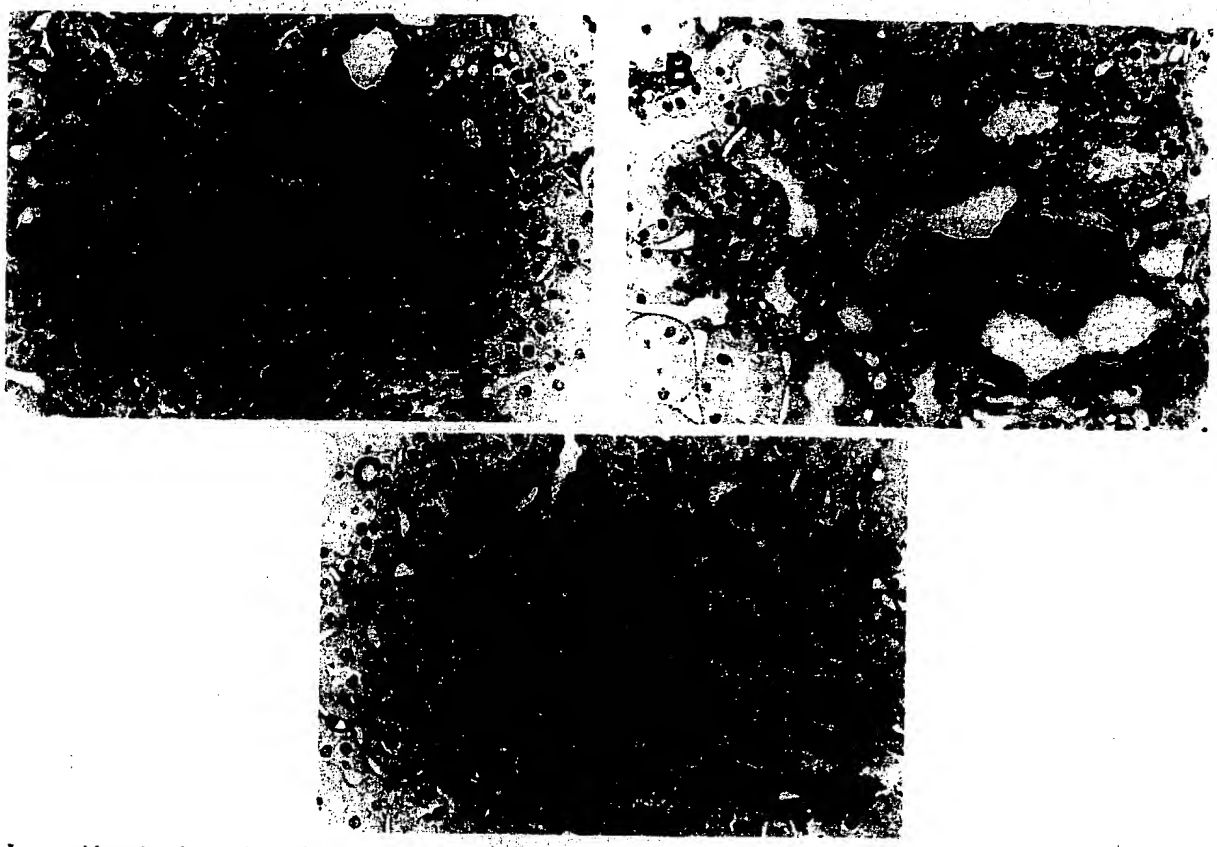

FIG. 7. Immunohistochemistry of type IV collagen in tubulointerstitium of control (*A*), diabetic (*B*), and ramipril-treated diabetic (*C*) rats. Increased immunostaining is present in kidneys from diabetic rats compared with control and ramipril-treated diabetic animals. Original magnification ×400.

epithelial and surrounding interstitial cells. In contrast, no such focal areas of injury were identified in control or ramipril-treated diabetic rats (Figs. 3 and 4). No hybridization was detected in sections probed with sense riboprobes for either TGF-β1 or α1 (IV) collagen (Figs. 3*D* and 4*D*).

Immunohistochemistry. Greater immunostaining for type IV collagen was present in the peritubular basement membrane of diabetic rats compared with control and ramipril-treated diabetic animals ($P < 0.05$) (Fig. 7 and Table 2). In diabetic rats, TGF-β immunostaining was found in greatest abundance in the renal cortex within epithelial cells of the proximal tubule and in association with focal tubular dilatation, where it was noted in both the tubular epithelium and in surrounding interstitial cells. Less intense TGF-β immunostaining was noted in distal tubules and within glomeruli of diabetic rats. In control and ramipril-treated diabetic rats, only sparse TGF-β protein was noted ($P < 0.05$ vs. diabetic rats) (Fig. 8). Tissues treated with normal rabbit IgG showed no positive staining (Fig. 9).

DISCUSSION

The present study demonstrated that experimental diabetes is associated with overexpression of TGF-β1 and α1 (IV) collagen in addition to histological evidence of tubulointerstitial injury. Blockade of the RAS by ACE inhibition resulted in amelioration of TGF-β1 and α1 (IV) collagen overexpression and associated structural and functional abnormalities.

Several studies of diabetic nephropathy in humans have documented a correlation between the extent of interstitial fibrosis and renal dysfunction (6,28,29), although the relative contributions of glomerular versus tubulointerstitial changes to disease progression remain uncertain (30). In vitro, high glucose concentrations stimulate collagen (31) and TGF-β (32) gene transcription in proximal tubular cells and, in vivo, diabetes is associated with tubular basement membrane (TBM) thickening. Indeed, the extent of TBM thickening correlates closely with mesangial expansion in patients with diabetic nephropathy (33).

Although severe glomerulosclerosis and tubulointerstitial fibrosis do not occur in the STZ-induced diabetic rat, this animal model does develop mesangial expansion, glomerular basement membrane thickening, and proteinuria (34). In addition, STZ-induced diabetes is accompanied by expansion of the TBM and mild tubulointerstitial changes (35). In the present study, increased TGF-β and α1 (IV) collagen mRNA were noted in tubular cells, particularly those of the proximal tubule from diabetic rats. In contrast, very low levels of gene transcription were noted in control and ramipril-treated diabetic rats. In the diabetic rats, the major histological abnormality noted in the tubulointerstitium was tubular dilatation. These abnormal areas were associated with abundant expression of TGF-β1 and α1 (IV) collagen by both tubular epithelial cells and surrounding interstitial cells. Such focal areas of tubular injury and intense expression of either TGF-β1 or α1 (IV) collagen were not seen in either control or ramipril-treated diabetic rats.

Previous reports of tubulointerstitial TGF-β and collagen gene overexpression have been confined to the first 4 weeks

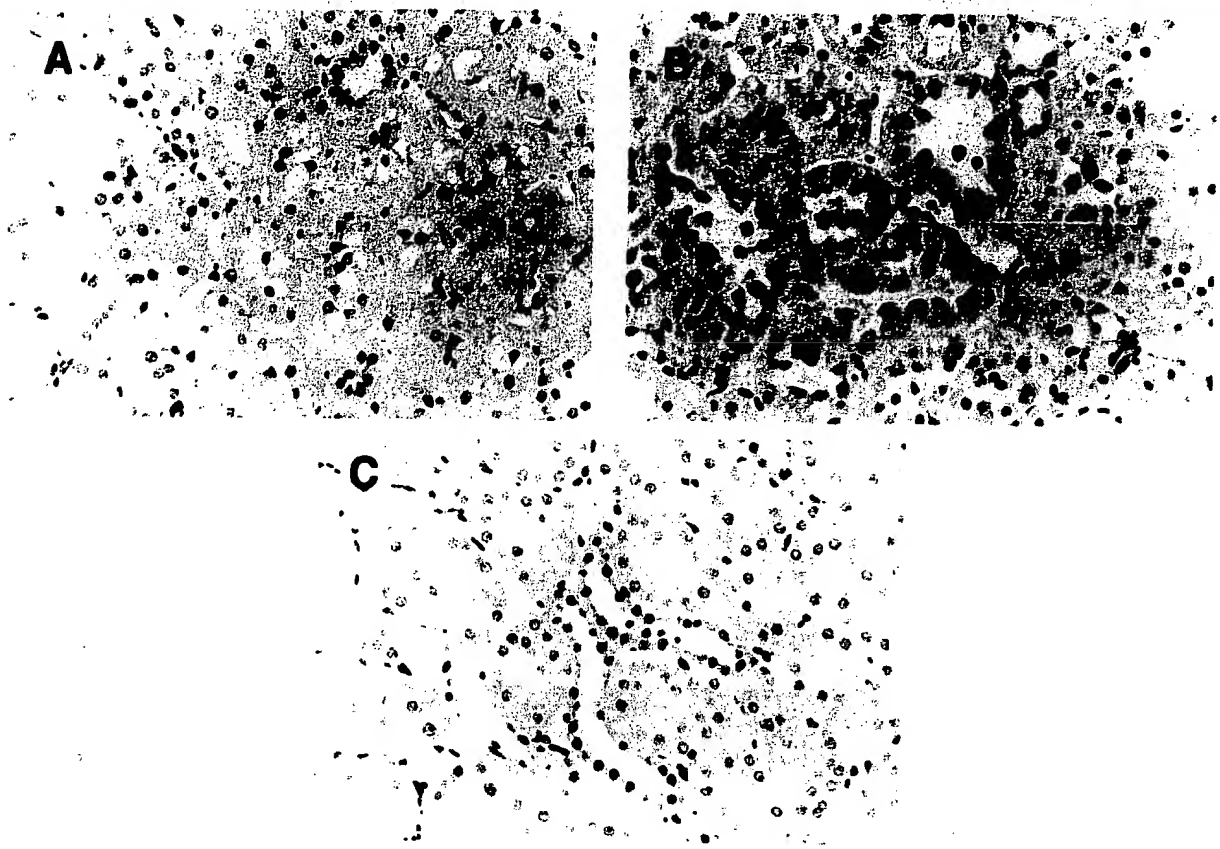

FIG. 8. Immunohistochemistry of TGF-β in tubulointerstitium of control (*A*), diabetic (*B*), and ramipril-treated diabetic (*C*) rats. Increased immunostaining is present in kidneys from diabetic rats compared with control and ramipril-treated diabetic animals. Original magnification ×400.

after the induction of experimental diabetes. For example, Ihm et al. (36) described increased expression of α1 (IV) collagen in proximal tubules of rats at 7 and 28 days after induction of diabetes with STZ. More recently, Park et al. (37) found increased tubulointerstitial TGF-β1 and α1 (IV) collagen mRNA at 3, 7, and 14 days of experimental diabetes and noted that these changes were ameliorated by insulin treatment. In the present study, overexpression of TGF-β1 and α1 (IV) collagen was found to persist at 6 months' duration of diabetes, suggesting that these gene transcripts may be involved in the pathogenesis of nephropathy rather than only during the acute phase of renal hypertrophy that follows the induction of experimental diabetes.

The described tubulointerstitial changes are unlikely to reflect a nephrotoxic effect of STZ for several reasons. First, the characteristic vacuolated tubular epithelial cells described in relation to STZ nephrotoxicity (37) were not observed in the present study. Furthermore, the finding of persistent overexpression of TGF-β and α1 (IV) collagen 6 months after induction of diabetes with STZ is not consistent with an acute toxic effect. Moreover, the ability of ramipril to prevent the described tubulointerstitial changes in untreated diabetic rats also argues against a primary toxic effect of STZ on tubules as a cause for the observed findings.

Several studies in both humans (15,38,39) and experimental animals (40,41) have demonstrated a renoprotective effect of ACE inhibition in diabetes. In diabetic rats, the ACE inhibitor enalapril has been shown to prevent the overexpression of glomerular ECM protein gene transcription (42). However, in other studies by the same investigators (43), enalapril failed to reduce the overexpression of TGF-β. By contrast, in the present study, treatment with ramipril was associated with amelioration of both enhanced TGF-β1 and α1 (IV) collagen expression. The reasons for the differing effects of ACE inhibition between our own study and that of Fukui et al. (43) are uncertain, particularly in the light of experimental evidence suggesting that ANG II may induce

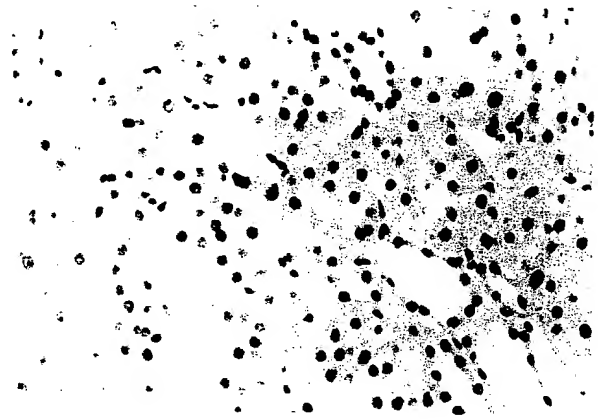

FIG. 9. Photomicrograph of kidney section treated with nonimmune rabbit anti-serum showing no positive staining. Original magnification ×400.

TGF-β expression (10). Certainly the role of the RAS in the pathogenesis of renal injury has been the subject of intense investigation. Although in some studies diabetes is associated with increased activity of the local intrarenal RAS (44,45), this has not been a universal finding (46,47). Such studies of the RAS in the diabetic kidney have involved the measurement of its constituents (e.g., its enzymes, substrates, effector molecules). A complete evaluation of the RAS would also require the measurement of postreceptor events and would be extraordinarily complex. Alternatively, a pharmacological approach could be used, as in the present study, whereby ACE inhibition is used as a means to effectively suppress ANG II–dependent pathways within the kidney (44). This approach has been previously shown to reduce collagen and TGF-β expression in both glomeruli and in the tubulointerstitium following subtotal nephrectomy (12), which, like diabetes, is associated with hyperfiltration, activation of the intrarenal RAS, and both glomerular and tubulointerstitial fibrosis (48). However, ANG II is not the only factor to be implicated in increased TGF-β expression in diabetes. Indeed, both glucose-dependent pathways, such as advanced glycation (26,49) and activation of protein kinase C (50), in addition to glucose-independent mechanisms, such as stretch and pressure (51), may lead to enhanced TGF-β transcription in diabetes. Thus the relative contribution of ANG II to TGF-β expression may differ between the glomerular and tubulointerstitial compartments. In addition, other fibrogenic cytokines may also contribute to ECM accumulation in diabetes, with differing expression at different sites within the kidney (52).

Although the present study demonstrated the presence of tubulointerstitial changes in experimental diabetes, it did not suggest that tubulointerstitial lesion is preeminent. Indeed, progressive tubulointerstitial injury in diabetes may well develop as a consequence of advancing glomerulopathy (30). This is consistent with suggestions that primary glomerular pathology leads to tubular injury by a number of pathogenetic mechanisms (1), although in the present study immunostaining for TGF-β was most marked in the proximal tubule rather than in the glomerulus. However, pathology at each of these sites may be important determinants of progressive renal dysfunction in diabetic nephropathy.

In summary, we found that experimental diabetes was accompanied by tubulointerstitial injury and associated overexpression of TGF-β1 and α1 (IV) collagen. Structural changes, increased TGF-β1 and α1 (IV) collagen gene transcription, and protein immunostaining were all ameliorated by treatment of diabetic rats with the ACE inhibitor, ramipril. The beneficial effects of ACE inhibition on the tubulointerstitium in diabetic renal disease may contribute to its therapeutic effect in diabetic nephropathy.

ACKNOWLEDGMENTS

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ISSN: 0012-1797.
DT Article; Journal
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LA English
REC Reference Count: 51
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Sean McGarry
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L6 ANSWER 26 OF 40 MEDLINE DUPLICATE 10
AN 1998184615 MEDLINE
DN 98184615 PubMed ID: 9525702
TI Link between angiotensin II and TGF-beta in the kidney.
AU Wolf G
CS Department of Medicine, University of Hamburg, Germany..
wolf@uke.uni-hamburg.de
SO MINERAL AND ELECTROLYTE METABOLISM, (1998) 24 (2-3) 174-80. Ref: 56
Journal code: 7802196. ISSN: 0378-0392.
CY Switzerland
DT Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
(REVIEW, TUTORIAL)
LA English
FS Priority Journals
EM 199805
ED Entered STN: 19980514
Last Updated on STN: 19980514
Entered Medline: 19980501

L6 ANSWER 35 OF 40 MEDLINE DUPLICATE 14
AN 96163237 MEDLINE
DN 96163237 PubMed ID: 8587237
TI ACE inhibition reduces proteinuria, glomerular lesions and extracellular matrix production in a normotensive rat model of immune complex nephritis.
AU Ruiz-Ortega M; Gonzalez S; Seron D; Condom E; Bustos C; Largo R; Gonzalez E; Ortiz A; Egido J
CS Renal Unit, Fundacion Jimenez Diaz, Universidad Autonoma, Madrid, Spain.
SO KIDNEY INTERNATIONAL, (1995 Dec) 48 (6) 1778-91.
Journal code: 0323470. ISSN: 0085-2538.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 199603
ED Entered STN: 19960404
Last Updated on STN: 19960404
Entered Medline: 19960327

ANSWER 36 OF 37 CAPLUS COPYRIGHT 2003 ACS
AN 1991:507031 CAPLUS
DN 115:107031
TI Transforming growth factor-.beta.1 up-regulates type IV collagenase expression in cultured human keratinocytes
AU Salo, Tuula; Lyons, J. Guy; Rahemtulla, Firoz; Birkedal-Hansen, Henning; Larjava, Hannu
CS Dep. Oral Surg., Univ. Oulu, Oulu, SF-90220, Finland
SO Journal of Biological Chemistry (1991), 266(18), 11436-41
CODEN: JBCHA3; ISSN: 0021-9258
DT Journal
LA English

L9 ANSWER 31 OF 37 BIOTECHNO COPYRIGHT 2003 Elsevier Science B.V.
AN 1993:23273871 BIOTECHNO
TI Interleukin-1.beta. and transforming growth factor-.alpha./epidermal

Kidney International, Vol. 48 (1995), pp. 1778–1791

ACE inhibition reduces proteinuria, glomerular lesions and extracellular matrix production in a normotensive rat model of immune complex nephritis

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ACE inhibition reduces proteinuria, glomerular lesions and extracellular matrix production in a normotensive rat model of immune complex nephritis. We studied the effect of the angiotensin converting enzyme (ACE) inhibitor, quinapril, on the clinical and morphological lesions of a normotensive model of immune complex nephritis. Untreated rats developed massive nephrotic syndrome, intense cell proliferation and glomerular and tubulointerstitial lesions. In the renal cortex of nephritic rats there was a significant increase in gene expression of TGF-β1, fibronectin and collagens, and ACE activity. Systolic blood pressure remained normal with progression of the disease. Administration of quinapril for three weeks to animals with glomerular lesions (proteinuria 20 to 50 mg/day) avoided the development of intense proteinuria (79 ± 28 vs. 589 ± 73 mg/day, $P < 0.001$) and decreased cell proliferation, glomerulosclerosis, tubulointerstitial lesions, and inflammatory infiltrates. Cortical gene expression of TGF-β1 and matrix proteins was also diminished. ACE activity was inhibited by 68% in renal cortex. These results show that quinapril administration to normotensive rats with immune complex nephritis decreases proteinuria and glomerular and tubulointerstitial lesions, probably modulating the local angiotensin II generation and its effects on cell growth, TGFβ and matrix protein synthesis.

The proliferation of glomerular cells and the additional production of matrix proteins have been associated with the onset of proteinuria, glomerulosclerosis and progressive renal failure in a large number of animal models [1, 2]. Although the precise mechanisms leading to matrix expansion are not known, there is evidence that growth factors and cytokines released by infiltrating leukocytes, platelets and resident glomerular cells play a significant role [1, 2].

Studies in the last few years have suggested that the renin-angiotensin system (RAS) may play a role in the pathogenesis of tissue damage [3, 4]. Early indications came from studies in which the treatment with ACE inhibitors reduced proteinuria and sclerosis in experimental models of nephritis, characterized by elevated systemic and glomerular capillary pressure, as the remnant kidney model and diabetic nephropathy [5–7]. Several investigators have attributed this beneficial effect to hemodynamic causes [5–9], while others have suggested that ACE inhibitors could have a primary effect on the permeability of the glomerular basement membrane [10–13]. Likewise, in chronic nephrosis induced by puromycin aminonucleoside, the administration of enalapril attenuated proteinuria, glomerular sclerosis and tubulointerstitial damage [14, 15].

The interest in the participation of RAS in tissue damage has recently increased. Angiotensin II (Ang II) promotes cell proliferation and synthesis of extracellular matrix proteins in different cells [16–18]. Furthermore, in renal cells, such as mesangial cells [19], tubular epithelial cells [20] and interstitial fibroblasts [21], Ang II induces expression and synthesis of growth factors which participate in tissue remodeling and sclerosis.

Although most human glomerular diseases are of immune origin, little information is available about the effect of ACE inhibitors on the evolution of immune complex-mediated glomerular injury. Nevertheless, recent reports have shown that ACE inhibitors may decrease proteinuria even in normotensive patients [22], suggesting the importance of local RAS in kidney injury.

For those reasons, the current study was designed to determine the effect of quinapril, an ACE inhibitor with intense tissular binding [23, 24], on a normotensive model of immune nephritis induced in rats by repeated injections of ovalbumin [25]. This model is characterized by heavy proteinuria, the presence of immune complexes in all glomerular areas, intense cellular proliferation and mesangial matrix expansion [25, 26]. We investigated whether the therapeutic effect of ACE inhibition involves attenuation of renal expression of transforming growth factor-β (TGF-β1) and extracellular matrix proteins in rats with nephritis. Finally, in order to assess the importance of renal ACE inhibition by quinapril, we determined the ACE activity in the kidney tissue.

Our results show that the administration of quinapril to rats with active nephritis decreased proteinuria, morphological lesions, and gene expression of TGF-β1 and extracellular matrix proteins. These data support the hypothesis that the local RAS could play an important role in the pathogenesis of the glomerular and interstitial damage in this model. This study provides compelling evidence for the use of ACE inhibitors in immune complex nephritis with overt glomerular injury, even in the absence of systemic hypertension.

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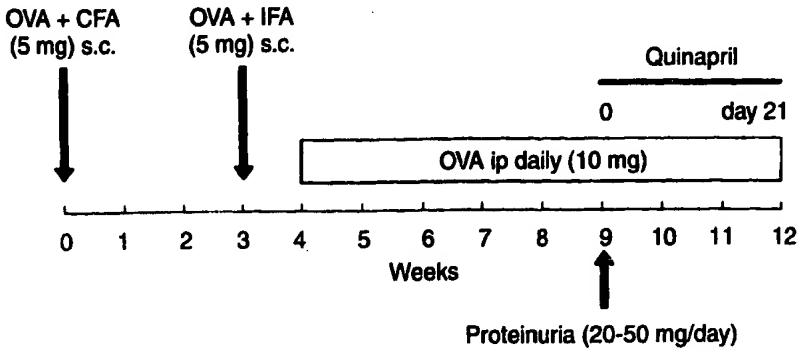

Fig. 1. *Schedule of experimental protocol for the induction of immune complex nephritis.* Immune complex nephritis was induced in normotensive rats. When proteinuria reached 20 to 50 mg/day animals were randomly distributed into two groups: quinapril-treated (33 ± 10 mg/day, N = 12) and untreated (30 ± 14 mg/day, N = 12; P = NS). After three weeks of study animals were sacrificed.

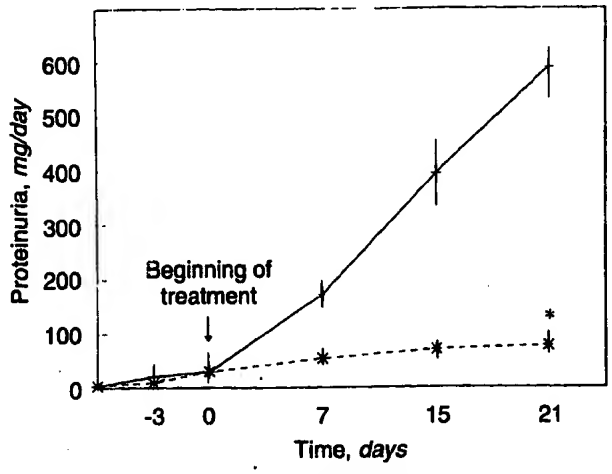

Fig. 2. *Urinary protein excretion rate during three weeks of observation.* Proteinuria in untreated (–) or quinapril-treated rats (···). Untreated rats developed massive proteinuria reaching the value of 589 ± 73 mg/day. Quinapril treatment prevented the development of intense proteinuria (79 ± 28 mg/day at 3 weeks, $^*P < 0.001$ vs. untreated rats). Proteinuria remained in the normal range (<5 mg/day) in healthy rats, treated or not with quinapril (not shown). Data are shown as mean ± SEM. N = 10 to 12 per group.

Table 1. Biochemical parameters

Group (N)	Total protein	Cholesterol	Creatinine
	g/dl	*mg/dl*	
Untreated nephritis (6)	4.6 ± 0.5[c]	330 ± 11[c]	1.00 ± 0.1[c]
Quinapril-treated nephritis (11)	5.9 ± 0.1[a]	161 ± 20[a]	0.9 ± 0.01[b]
Healthy controls (12)	6.5 ± 0.3	65 ± 13	0.8 ± 0.1
Quinapril controls (12)	6.5 ± 0.3	65 ± 22	0.8 ± 0.07

Results are expressed as mean ± SEM.
[a] $P < 0.005$, [b] $P < 0.01$ Quinapril-treated versus untreated nephritic, [c] $P < 0.005$, untreated versus healthy controls

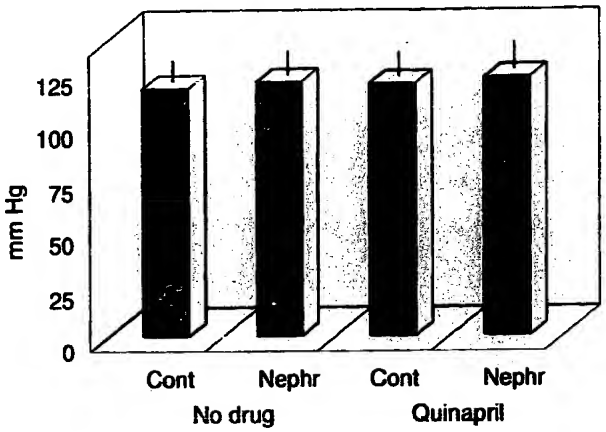

Fig. 3. *Blood pressure measurement in all groups of animals.* Systolic blood pressure remained stable in all groups. Bars represent mean ± SEM at the end of the period of study. N = 5 to 6 per group.

Methods

Experimental design

Studies were conducted in normotensive female Wistar rats with initial weights of 200 to 220 g (obtained from the Fundación Jimenez Díaz animal facilities). Immune complex nephritis was induced according to a previously described protocol [25, 26], shown in Figure 1. Briefly, rats received an initial subcutaneous injection of 5 mg of ovalbumin (OVA, Sigma Chemicals, St. Louis, MO, USA) in complete Freund's adjuvant (CFA) (Difco, Detroit, MI, USA), and three weeks afterwards, the same dose was given in incomplete Freund's adjuvant (IFA; Difco). One week later, daily intraperitoneal administration of 10 mg ovalbumin was started. Proteinuria appeared around the 9th week. When proteinuria reached 20 to 50 mg/day, animals were randomly distributed into two groups:

Untreated group. Animals with spontaneous development of nephritis.

Quinapril-treated. Animals treated with the ACE inhibitor quinapril (as powdered hydrochloride, a gift from Parke Davis, Barcelona, Spain) at a concentration of 100 mg/liter, added to the drinking water and replaced every 48 hours.

Three weeks after the onset of proteinuria, animals were sacrificed, blood collected and kidneys removed. A parallel control group of animals of the same age, with or without treatment, was also studied.

Biochemistry

Urine was collected periodically from rats housed for 24 hours in metabolic cages with access to water only. Proteinuria was measured by the sulfosalicylic acid method.

Fig. 4. *Effect of quinapril treatment on glomerular and tubulointerstitial damage.* **(A)** Microphotograph shows glomerulus from a nephritic rat that has been stained with Masson trichrome. Note the expansion of the mesangial area, cellular proliferation and sclerotic lesions. **(B)** In contrast, the glomerulus from a quinapril-treated rat shows a slight increment in the mesangium. Magnification ×600.

At the end of the study period, serum levels of creatinine, total protein and cholesterol were determined according to standard methods. Creatinine clearance was calculated from three urine samples taken 72 hours before the animal was sacrificed.

Blood pressure measurement

Systolic arterial blood pressure was measured in conscious, restrained rats by a tail-cuff sphygnomanometer (NARCO Biosystems, CO, USA). The blood pressure value for each rat was calculated as the average of three separate measurements at each session.

Kidney tissue processing

At the time of sacrifice, the animals were fasted overnight and anesthesized with 5 mg/100 g sodium pentobarbital. The kidneys were perfused *in vivo* via the abdominal aorta with 100 ml of normal saline at 4°C, while the left renal vein was punctured to permit the perfusate to drain [27]. Blood samples were collected, centrifugated (2000 g for 10 min) and aliquots were stored at −80°C for ACE activity determination. The kidneys were removed immediately and further processed for histological studies, ACE determination and RNA extraction.

Renal histopathological studies

All histological studies were performed without the observer knowing to which group the animals belonged (double blind study).

Tissue for light microscopy was fixed in buffered formalin and embedded in paraffin. Sections (2 to 3 μm thickness) were prepared and stained with hematoxylin-eosin, Masson trichrome and periodic acid-Schiff. Glomerulosclerosis was defined as the disappearance of cellular elements from the tuft, the collapse of capillary lumens, and/or the folding of the glomerular basement membrane with entrapment of amorphous material. Mesangial

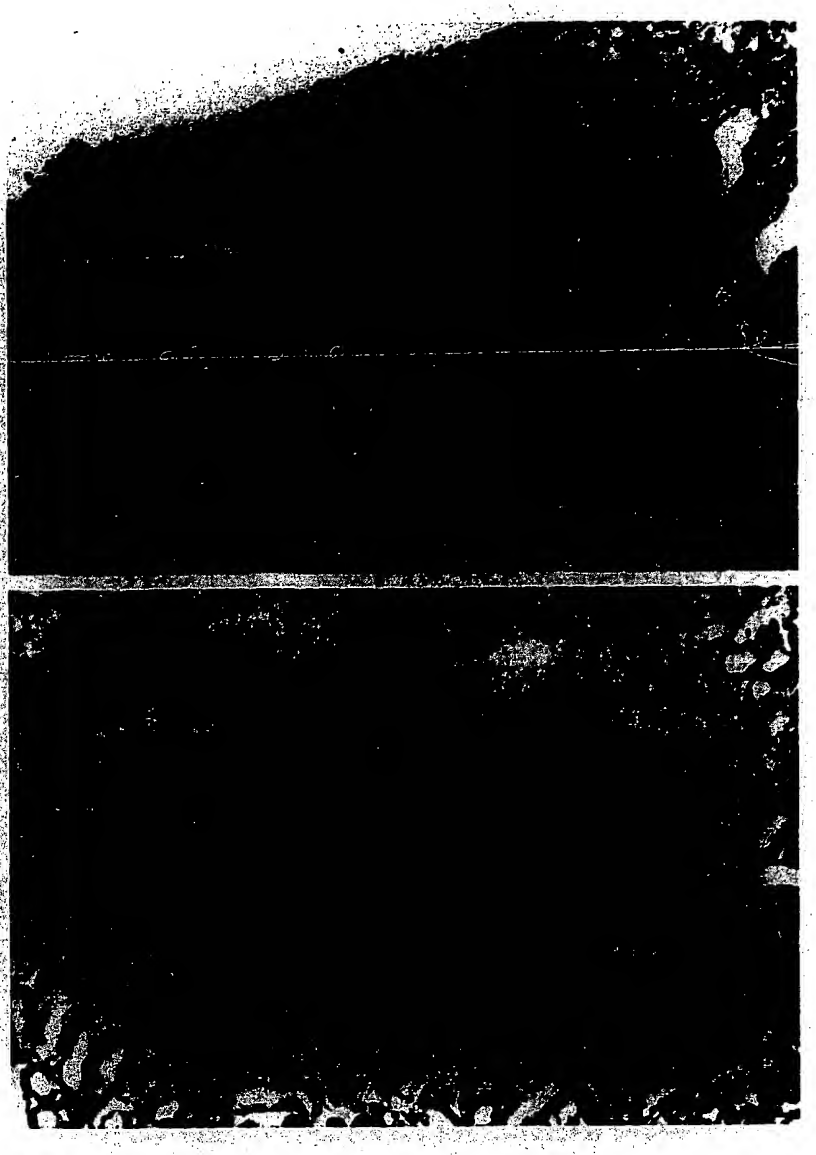

Fig. 4. (C) A representative microphotograph of an untreated rat stained with hematoxylin-eosin. Note the flattening of tubular cells, tubular dilation and presence of protein casts. In addition, an increase in mononuclear cell infiltration was present in the interstitium. (D) Almost normal tubulointerstitial morphology was observed in quinapril-treated rats. Magnification ×100.

expansion was defined by the presence of increased amounts of material in the mesangial region that reacted positively with a periodic acid-Schiff stain.

The glomerular immune deposits were semiquantitatively scored, as previously described [28]: 0+, none; 1+, isolated mesangial deposits; 2+, mesangial deposits and subendothelial deposits in less than 50% of the glomeruli; 3+, mesangial deposits and subendothelial deposits in more than 50% of the glomeruli.

Tissue for immunohistochemistry and immunofluorescence was embedded in OCT (Tissue-Tek, Miles, Elkhart, IN, USA) snap-frozen in liquid nitrogen and stored at −80°C until the study.

The quantification of the infiltrating glomerular and interstitial cells was performed by using an indirect immunoperoxidase technique [29]. Only the cells with a nucleus that could be clearly identified were counted. The counting was done by magnifying the images. In the interstitium, areas of 0.45 mm^2 were counted. The mean number of cells per glomerular cross section was determined by evaluating 50 glomeruli in each renal section. The monoclonal antibodies employed were the following: 0×1 (leukocyte common antigen, LCA), W3/13 (T lymphocytes, LT) (Seralab, Sussex, UK) and ED1 (monocytes/macrophages) (Serotec, Oxford, UK).

To examine the distribution and intensity of fibronectin and collagens, indirect immunofluorescence was employed. Frozen tissues were sectioned at 4 μm thickness using a cryostat. Air-dried sections were fixed in cold acetone and incubated with antibodies against extracellular matrix proteins. Primary antibodies used were rabbit anti-rat fibronectin [30] and anti-rat collagen type III (Chemicon, Temecula, CA, USA), and goat anti-rat collagen type IV (UNLB, Birmingham, UK). The secondary antibodies were FITC-labeled goat anti-rabbit IgG or FITC-labeled donkey anti-goat IgG (Binding Site, Birmingham, UK). As control experiments, tissue sections were incubated with normal rabbit serum, followed by FITC-labeled antibodies or secondary antibody alone. The slides were evaluated according to a semiquantitative score, as previously described [29].

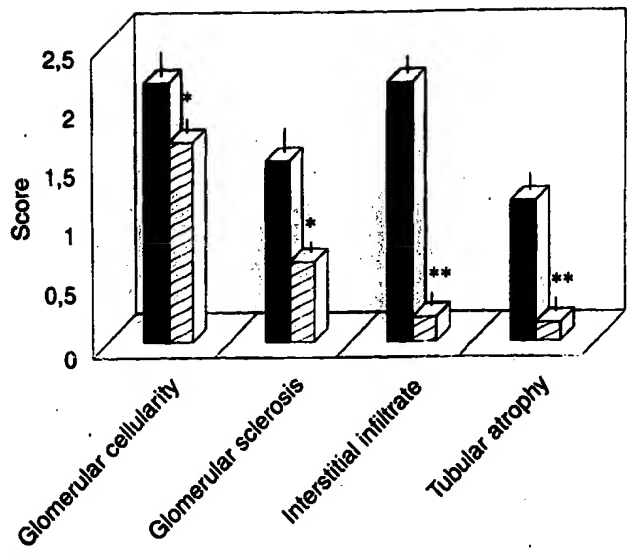

Fig. 5. *Semiquantitative analysis of glomerular and tubular damage in rats with nephritis.* Bars represent semiquantitative score of 6 untreated (■) or quinapril-treated (▨) rats. The values are expressed as mean ± SEM. $^*P < 0.05$; $^{**}P < 0.005$. There was no difference between healthy-controls and quinapril-treated control rats (not shown).

To localize the immune deposits rabbit FITC-labeled anti-rat IgG (Binding Site) was used for direct immunofluorescence.

Kidney mRNA isolation and hybridization studies

Tissue was divided in cortex and medulla, snap-frozen in liquid nitrogen and stored at −80°C until study. Kidney tissue was homogenized with a politron. RNA was obtained by the acid guanidine-thiocyanate-phenol-chloroform method [31] and quantified by absorbance at 260 nm in duplicate.

For quantitative studies, slot-dot hybridization was performed with serial dilutions of total RNA after formaldehyde denaturation. Four concentrations from each sample (serial dilution 1:2 from 40 μg) were blotted to nylon filters using a slot-dot apparatus. RNA was fixed by baking at 80°C for 90 minutes [32].

For Northern blot analysis, equal amounts of RNA (40 μg) were denatured and electrophoresed in a 1% agarose-formaldehyde gel and transferred to nylon membranes (Genescreen, New England Nuclear, Boston, MA, USA). By means of ethidium bromide staining we determined in each gel the equivalent loading of RNA and its absence of degradation, the position of the 28S and 18S ribosomal RNA, and the efficacy of capillary transfer. RNA was fixed to the nylon membrane by baking at 80°C for 90 minutes.

The cDNA probes used were (α1)I (Hf677), (α1)III (Hf939) and (α1)IV (pCVIV-1-PE16) collagens (obtained from American Type Culture Collection, Rockville, MD, USA). Probes were prepared as described previously [31] and radiolabeled by nick translation method (Boehringer Mannheim, Germany) with α-^{32}[P]dCTP (DuPont, Boston, MA, USA). The cDNA probes of rat fibronectin (SR270), used as 270 bp *Eco*RI fragment (provided by R.O. Hynes, Massachusetts Institute of Technology [33]) and murine TGF-β1, used as a 279 bp *Eco*RI fragment (a gift from F.N. Ziyadeh, University of Pennsylvania [34]) were radiolabeled by random primer method (Boehringer Mannheim) with α-^{32}[P]dCTP.

The membranes were prehybridized at least for four hours at 42°C in 50% formamide, 1% SDS, 5 × SSC, 5 × Denhardt's solution, 0.25 mg/ml denatured salmon sperm DNA and 50 mM sodium phosphate buffer pH 6.5. Hybridization was carried out at 42°C overnight with 20% dextran sulfate and α-^{32}[P]-denatured probe. The filters were washed using a 2 × SSC, 0.1% SDS, for 30 minutes at room temperature and then twice with 0.2 × SSC, 0.1% SDS, at 55°C for 15 minutes. Blots were reutilized by stripping. Autoradiography was performed by standard methods.

Autoradiography films were scanned using the Image Quant densitometer (Molecular Dynamics, Sunnyvale, CA, USA). To determine the efficiency of dot blot experiments, a lineal regression analysis was performed from the data obtained using only those where the correlation was $r = 0.99$. For quantitative comparisons, the density of the bands was corrected by the density of 28S at the same dilution. The resulting values were then divided by the values for control animals. Therefore the data presented show the fold change of mRNA relative to control animals.

Measurement of ACE activity

Tissue for ACE activity determinations was dissected in cortex and medulla, frozen in liquid nitrogen and stored at −80°C until its study. Samples were homogenized in distilled water and centrifuged at 12000 g for 10 minutes at 4°C. The resulting supernatant was used for analysis of tissue ACE activity.

The glomeruli were isolated using the graded sieving technique [35], washed twice in PBS, and resuspended in distilled water. Samples were homogenized and spun down at 12000 g for five minutes at 4°C. The resulting supernatant was used for analysis of ACE activity. All glomerular preparations used consisted of more than 95% glomeruli with minimal tubular contamination.

The brush border membranes were isolated from renal cortex, using the method of Malathi et al [36], based on calcium aggregation followed by differential centrifugation. The final pellet containing the brush borders was resuspended and diluted in 0.3% Triton X-100 for enzyme activity measurement and was then stored at −80°C. The brush border preparation was characterized by dosing specific enzyme markers [36].

The ACE activity was determined by a spectrophotometric method (Sigma) based on the enzymatic reaction catalyzed by ACE, where the furilacylphenylanylglycilglycine (FAPGG) was hydrolized to furacylphenylalanine (FAP). The FAPGG hydrolysis produced a decrease in the absorbance at 340 nm, which was directly proportional to the ACE activity of the sample. ACE activity is expressed as relative units per milligram protein of tissue and brush border membrane, as determined by the Lowry method [37] and as units per ml in serum.

Statistical analysis

ANOVA and unpaired Student's *t*-test were used for proteinuria, serum creatinine, serum total protein, serum cholesterol, ACE activity and blood pressure measurements. The Kolmogorov-Smirnov test was used to assess the normal distribution of the data. The Mann-Whitney U-test for nonparametric values was used for semiquantitative variables. Differences were considered significant if the *P* value was less than 0.05. Results are expressed as mean ± SEM.

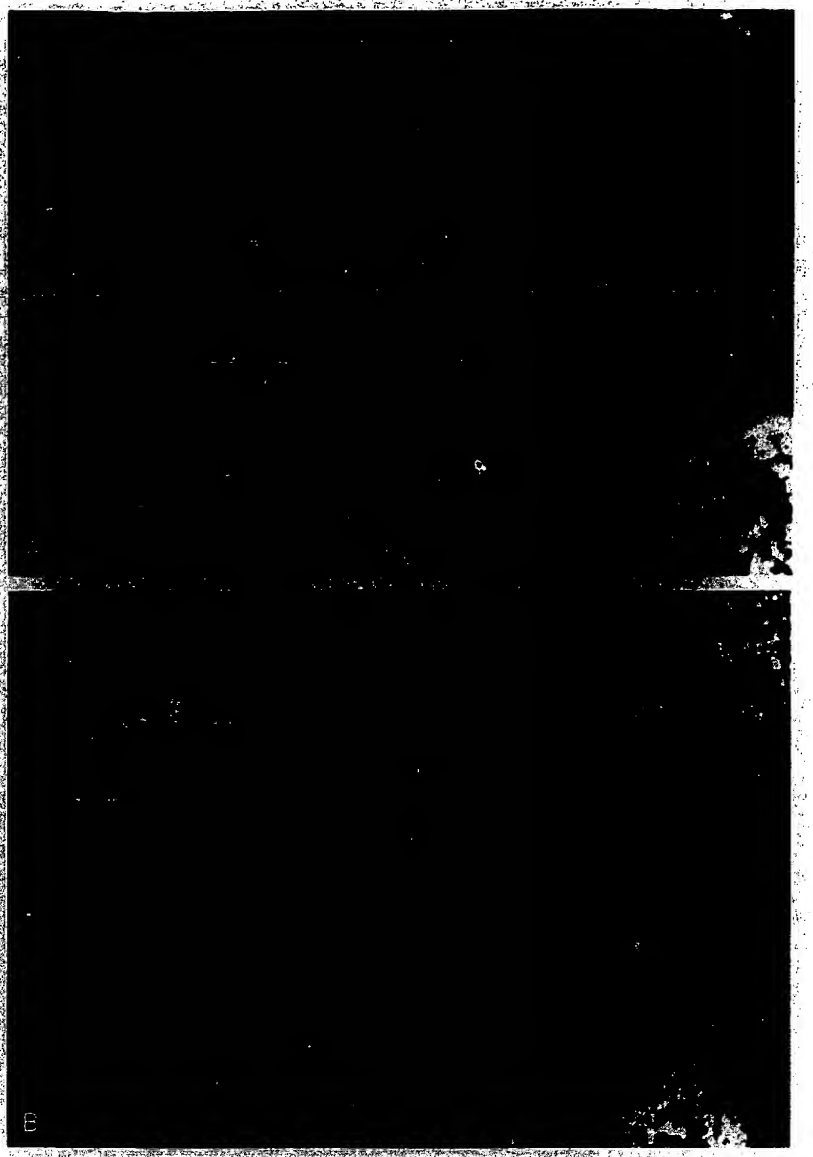

Fig. 6. *Localization of immune deposits by immunofluorescence in nephritic rats.* **(A)** In untreated rats, IgG deposits were localized in the glomerular mesangium and capillary walls. **(B)** There was no change in the localization of immune deposits in response to treatment.

Results

Evolution of disease

In this model of immune complex nephritis proteinuria appears four to five weeks after intraperitoneal injection of ovalbumin, and approximately three weeks later the animals develop nephrotic syndrome and renal failure. At this time, glomerular immune deposits are abundant both in mesangium and in all areas of the capillary wall. There is also intense glomerular hypercellularity with segmental fibrinoid necrosis and mononuclear cell infiltration in the glomeruli and in the renal interstitium [25]. When proteinuria reached values between 20 to 50 mg/day animals were randomly distributed into two groups: treated (33 ± 10 mg/day) and untreated (30 ± 14 mg/day; P = NS).

Evolution of proteinuria and renal function

The administration of quinapril (100 mg/liter in the drinking water) avoided the development of intense proteinuria. At the end of the study (21 days of treatment), the proteinuria in the quinapril-treated rats was significantly reduced versus untreated rats (79 ± 28 vs. 589 ± 73 mg/day; $P < 0.001$; Fig. 2). The untreated nephritic animals developed renal failure compared to healthy rats (creatinine clearance 236 ± 52 vs. 307 ± 21 μl/min/100 g; $P < 0.01$). In rats treated with quinapril, the renal function improved with respect to nephritic rats (310 ± 60 μl/min/100 g).

Biochemical parameters

Rats with nephritis presented hypoproteinemia and hypercholesterolemia (Table 1). Rats treated with quinapril showed a significant increase in total serum proteins and a significant decrease in serum cholesterol levels compared to untreated rats (Table 1).

Blood pressure

Mean systolic blood pressure in all rats was in the normotensive range throughout the three weeks of study. No change in systolic blood pressure in nephritic rats (119 ± 7 mm Hg) versus controls

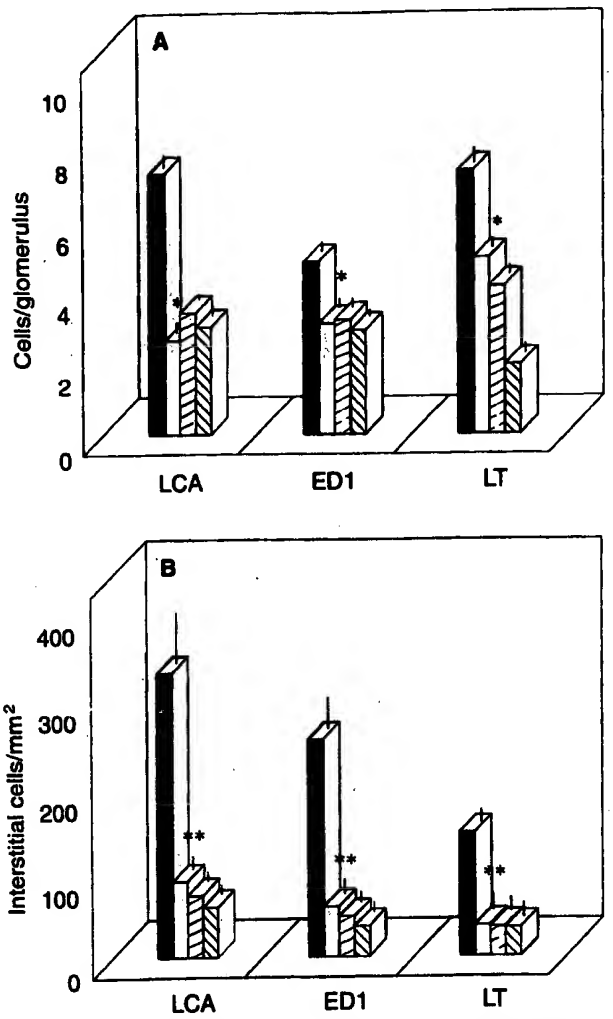

Fig. 7. *Mononuclear cell infiltration in the glomeruli and interstitium.* The quantitation of the infiltrating glomerular (A) and interstitial (B) cells was performed by immunoperoxidase technique. Bars represent untreated nephritis (■), quinapril-treated nephritis (□), healthy-control (▨) and quinapril-treated control rats (▧). The values are expressed as mean ± SEM. $N = 5$ to 6 per group. $^*P < 0.05$; $^{**}P < 0.005$ quinapril versus untreated rats.

(118 ± 7 mm Hg) was noted. Treatment with quinapril did not modify the systolic pressure either in rats with nephritis (117 ± 11 mm Hg) or in healthy controls (115 ± 8 mm Hg; P = NS; Fig. 3).

Morphological studies

Previous studies from our group have detailed the morphological aspects of glomerular lesions [25]. In brief, in untreated rats there was glomerular hypercellularity, mesangial matrix expansion, segmental fibrinoid necrosis, glomerulosclerosis, and mononuclear cell infiltration in the glomeruli and in the interstitium (Figs. 4 and 5). After three weeks of treatment with quinapril, we noted a significant decrease in the glomerular cellularity, matrix expansion, glomerular sclerosis, interstitial infiltrates and tubular atrophy (Figs. 4 and 5). A positive linear correlation was found between the glomerular sclerosis, tubular atrophy and proteinuria ($P < 0.005$).

This nephritis is characterized by the presence of immune deposits in all glomerular areas [25]. Immune complexes can stimulate mesangial cells proliferation and extracellular matrix synthesis, through the activation of cytokine and growth factor synthesis [38, 39]. Quinapril treatment reduced slightly, but not significantly, the glomerular immune deposit score. Nevertheless, deposits were still observed in the mesangium and glomerular capillary wall in both groups of animals (2.0 ± 0.6 quinapril-treated vs. 2.3 ± 0.4 untreated rats; light microscopy, semiquantitative score, P = NS). Furthermore, immunofluorescence studies showed that there was no change in the localization and intensity of IgG deposits (Fig. 6). Although previous studies have demonstrated that Ang II modifies mesangial trafficking of macromolecules [40], the absence of a significant reduction in the amount of immune deposits in treated animals suggests that the beneficial effect of quinapril is not due to this mechanism.

We also studied the phenotype of glomerular and interstitial inflammatory cellular infiltrates by monoclonal antibodies and indirect immunoperoxidase technique. Rats treated with quinapril for three weeks had less glomerular total leukocytes (OX1, 2.7 ± 0.9 vs. 7.5 ± 0.4, cells/glomerulus, $P < 0.05$), T lymphocytes (W3/13, 3.2 ± 0.7 vs. 5.0 ± 1.1, $P < 0.05$) and monocytes/macrophages (ED1, 5.1 ± 1.9 vs. 7.6 ± 0.1, $P < 0.05$) than untreated rats with nephritis (Figs. 7A and 8). The effect of quinapril on the interstitial cellular infiltrates was more marked, with important reduction in the number of leukocytes (91 ± 14 vs. 343 ± 180 cells/mm² untreated rats, $P < 0.005$), T lymphocytes (60 ± 9 vs. 262 ± 45, $P < 0.005$) and macrophages (37 ± 8 vs. 149 ± 14, $P < 0.005$) that approached normal values (Fig. 7B).

Extracellular matrix proteins

In normal kidney, fibronectin is present in the mesangium and along the glomerular basement membrane. During glomerular injury increased fibronectin and collagen IV deposition is frequently observed [41, 42].

In animals with nephritis, the immunofluorescence studies showed that fibronectin and collagen type IV increased in the mesangium with extension to the capillary wall, and appeared in the tubular basement membrane and in interstitium (Fig. 9). In quinapril-treated rats, an important diminution in the glomerular deposition of both proteins was noted (Fig. 9). By contrast, with this semiquantitative technique, no clear differences were found in interstitial collagens in response to treatment (data not shown).

In the renal cortex of untreated nephritic rats, dot blot studies showed a 25-fold increase in fibronectin mRNA, and an eightfold increase in collagen type IV mRNA, as compared to healthy rats (Fig. 10). The mRNA of interstitial collagens type I and III was also increased, although to a lesser extent (3.7-fold and 2.8-fold, respectively; Fig. 10). The increase in matrix proteins mRNA in the kidney, roughly correlated with the findings on immunofluorescence, indicates that the accumulation of renal matrix proteins was due to increased gene transcription, as occurs in other renal diseases [43].

The treatment with quinapril down-regulated the expression of fibronectin, and collagens IV, I and III (Fig. 10). Compared to untreated nephritis, collagen type IV showed the greatest decrease (86%), followed by fibronectin (65%) and the interstitial collagens I and III (both around 40%).

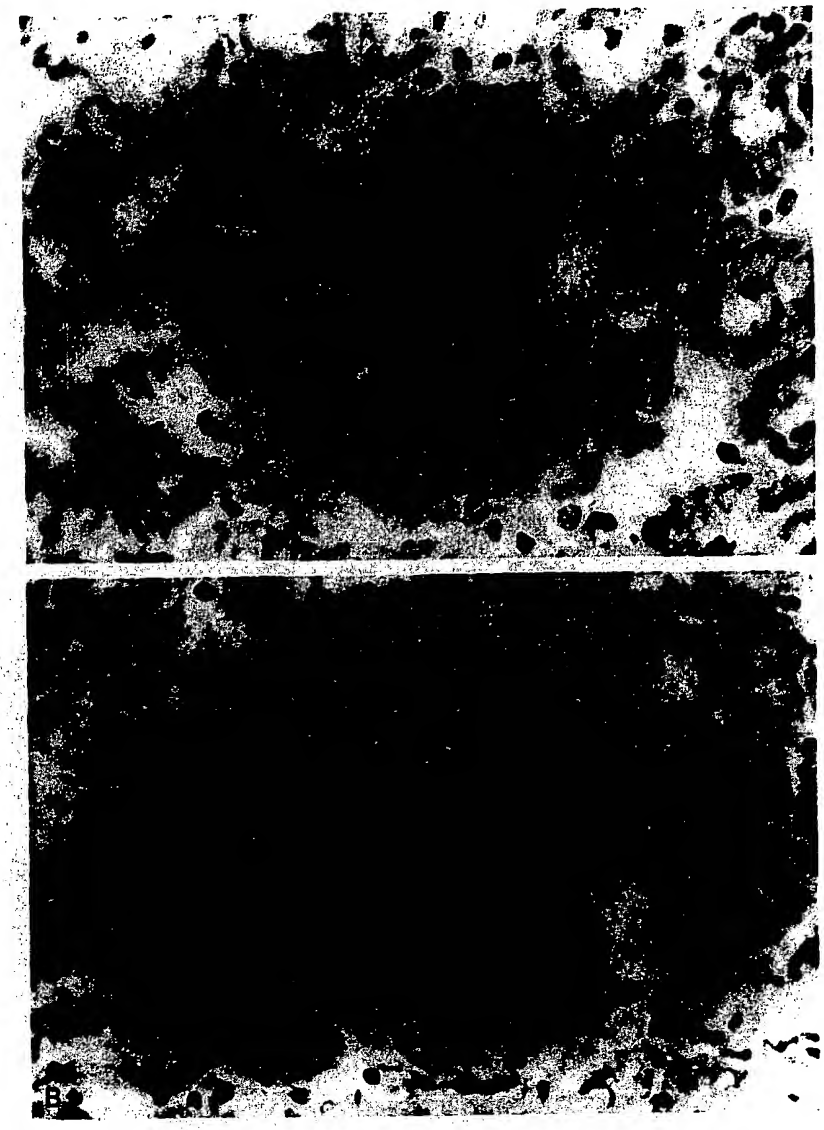

Fig. 8. *Effect of quinapril on glomerular infiltration by macrophages.* Representative microphotographs of immunoperoxidase staining sections. **(A)** Note intense glomerular infiltration by macrophages (ED1+ cells) in untreated rat. **(B)** Discrete presence of macrophages in the glomerulus of a quinapril-treated animal.

Renal TGF-β1 mRNA expression

In experimental nephritis, a close association between elevated TGF-β1 mRNA expression and the development of glomerular injury has been described [44]. A Northern blot analysis of cortical TGF-β1 mRNA expression in control rats showed a weak 2.5 Kb band (Fig. 11), suggesting that there was a low level of expression of this growth factor in the cortex. In nephritic rats, there was a marked increase in cortical TGF-β1 mRNA, which decreased in quinapril-treated rats. By contrast, there was no increase in medullary TGF-β1 mRNA (data not shown). Dot blot analysis showed similar results (Fig. 11).

ACE activity in serum and kidney

Serum ACE activity of rats with nephritis did not differ from controls (Fig. 12A). ACE activity in the cortex of rats with nephritis was significantly elevated compared to control rats ($P < 0.005$; Fig. 12B). These results suggest a different behavior between circulating and tissue renal RAS in this model.

In a parallel group of animals with nephritis, we determined ACE activity in the glomeruli and in the brush border membrane of the renal cortex. Glomerular ACE activity did not differ from controls. By contrast, brush border ACE activity, that was about 100 times higher than in the original cortical homogenates, was elevated compared to controls ($P < 0.05$; Fig. 12C), suggesting that the observed increase in cortex ACE activity was chiefly due to the brush border membrane. ACE activity in medulla increased in a more modest manner, not reaching statistical significance (Fig. 12B). This suggests that ACE activity is regulated according to location in renal tissue.

In response to quinapril, serum ACE was completely inhibited (96%; Fig. 12A). ACE activity in the cortex and renal medulla was inhibited by 68 and 79%, respectively, in response to quinapril treatment (Fig. 12B).

Discussion

The beneficial effect of ACE inhibitors on the progression of renal disease has been demonstrated in different animal models associated or not to hypertension [6, 7, 16], but the precise

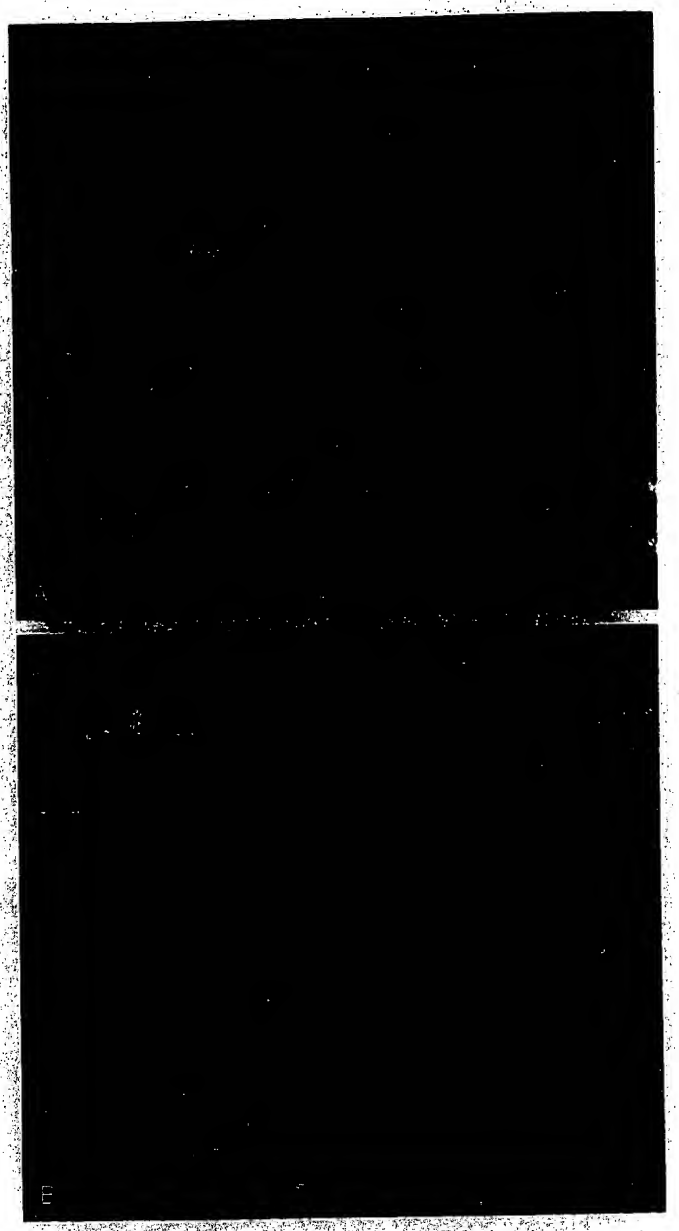

Fig. 9. *Immunofluorescence detection of extracellular matrix components.* In normal rats, fibronectin was deposited in mesangial areas. Note an intense staining in the glomerulus of nephritic rats (A), which diminished in quinapril-treated rats (B).

mechanism of their actions remains unknown. In this work, we have studied the effect of ACE inhibition in a normotensive model of glomerular immune injury, characterized by immune deposits in all glomerular areas that morphologically resemble human mesangiocapillary glomerulonephritis and active lupus nephritis [25, 26].

In this model, untreated nephritic rats developed massive nephrotic syndrome and renal failure approximately three weeks after the onset of proteinuria [26], without modification of blood pressure. The administration of quinapril, an ACE inhibitor with strong tissue enzyme binding, to animals with established nephritis resulted in significantly less proteinuria and preservation of renal function.

Progressive renal scarring is associated with glomerular and interstitium infiltration by inflammatory cells [1]. In untreated nephritic rats, we observed infiltration of macrophages and T cells. In animals treated with quinapril there was a significant reduction in glomerular and interstitial infiltration. This effect may be one of the mechanisms that ameliorates the evolution of renal sclerosis. These results could be explained by the modification of the proliferative and chemoattractant effects of Ang II [17]. In this sense, *in vivo* infusion of Ang II induced a marked vascular, glomerular and tubulointerstitial damage with increased proliferation of renal resident cells and inflammatory cell recruitment [45].

As it occurs in other progressive glomerulonephritis [1, 2], in this model of immune nephritis, untreated rats presented an accumulation of extracellular matrix in the glomeruli and the interstititum. A marked increase in mRNA expression of normal constituents of the glomerular extracellular matrix and of interstitial collagens was noted. By immunofluorescence, an increase in the glomerular and interstitial deposition of the two matrix proteins studied (fibronectin and type IV collagen) was also observed. In nephritic rats receiving quinapril there was a diminution in the mesangial expansion and glomerular sclerosis, accompanied by a marked down-regulation in the renal cortex gene expression of fibronectin and collagen IV. These results suggest that the beneficial effect of ACE inhibition on extracellular matrix synthesis may occur at the transcriptional level. In quinapril-treated rats the tubulointerstitial area was almost normal. The protection conferred by quinapril could be secondary to the diminution of proteinuria or to the inhibition of the local components of the RAS, as occurs in models of interstitial fibrosis [14, 46]. These data are in agreement with those observed in the unilateral ureteral ligation model, in the sense that treatment with enalapril dramatically diminished the interstitial fibrosis and the gene expression of matrix proteins [46].

TGF-β is a multifunctional cytokine that plays a major biological role in the regulation of extracellular matrix deposition [47, 48]. TGF-β gene expression is increased in several models of acute and chronic renal injury in parallel with extracellular matrix expansion [49]. Recent studies have stressed the interaction of RAS and TGF-β. Ang II induces TGF-β mRNA expression in renal cells [19–21]. The *in vivo* subcutaneous infusion of Ang II led to increased glomerular TGF-β gene expression [19]. These data suggest that Ang II directly induces TGF-β, which in turn induces fibrotic changes. In our experimental model, the maximal glomerular and tubulointerstitial damage coincided with an augmentation in TGF-β1 mRNA expression in the renal cortex. It must be highlighted that the treatment with quinapril normalized TGF-β1 gene expression. These data indicate that the observed therapeutic effect on synthesis of extracellular matrix components may be due to reduced TGF-β1 gene expression.

An important finding of our work was that the beneficial effect of quinapril occurred in the absence of high blood pressure. Contrasting with the normal values of serum ACE activity in rats with nephritis, a marked elevation of ACE activity was noted in the renal cortex of those animals, chiefly in the brush border membranes of renal proximal tubules, suggesting a local activation of RAS. The components of RAS have been found modified in different models of renal injury as diabetic nephropathy, obstruction nephropathy and uninephrectomized rats with high protein intake [50–52]. In response to the treatment, serum ACE

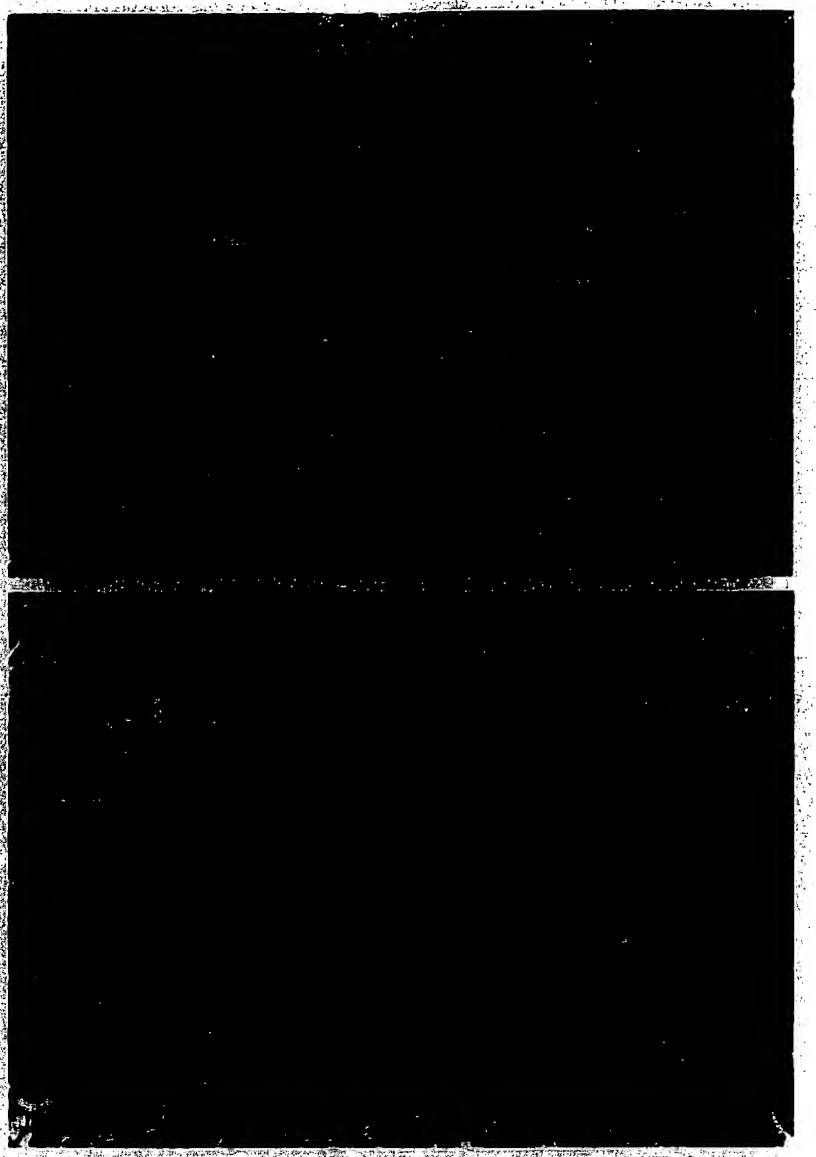

Fig. 9. (continued) In nephritic rats, type IV collagen is found along the glomerular basement membrane, in the mesangial matrix as well as in the basement membrane of Bowman capsule and in the tubuli in almost equal amounts (C). Note that there is an increase in type IV collagen deposition in mesangial matrix of the glomerulus, that decreases in quinapril-treated rats (D).

activity was completely suppressed in quinapril-treated rats. The importance of inhibiting ACE activity in tissue rather than in serum has recently been emphasized [3, 4]. At renal level ACE activity was reduced by quinapril therapy between 60 to 80%, to the same extent as observed by other authors employing similar doses of quinapril in the neointima after vascular injury [53].

Since ACE inhibition, besides reducing Ang II formation, also increases bradykinin concentrations, with the subsequent generation of nitric oxide and prostanglandins, the possibility exists that other mechanisms, independently of Ang II actions, could contribute to the beneficial effect of ACE inhibitors. Further studies employing Ang II or bradykinin receptor antagonists may be necessary to solve this point.

In summary, we have demonstrated that, in a normotensive model of immune nephritis, the administration of an ACE inhibitor attenuates the development of massive proteinuria, amelioriates the morphological lesions, and decreases the gene expression of TGF-β1 and matrix proteins. The inhibition of the Ang II actions on resident cell proliferation and matrix protein synthesis, via TGF-β, may be another mechanism of the beneficial effect of these drugs in immune and nonimmune glomerular injury, independently of the presence of high blood pressure. These data, together with those traditionally known about the glomerular hemodynamic improvement by ACE inhibitors, provide additional evidence for the employment of these drugs in patients with progressive renal disease.

Acknowledgments

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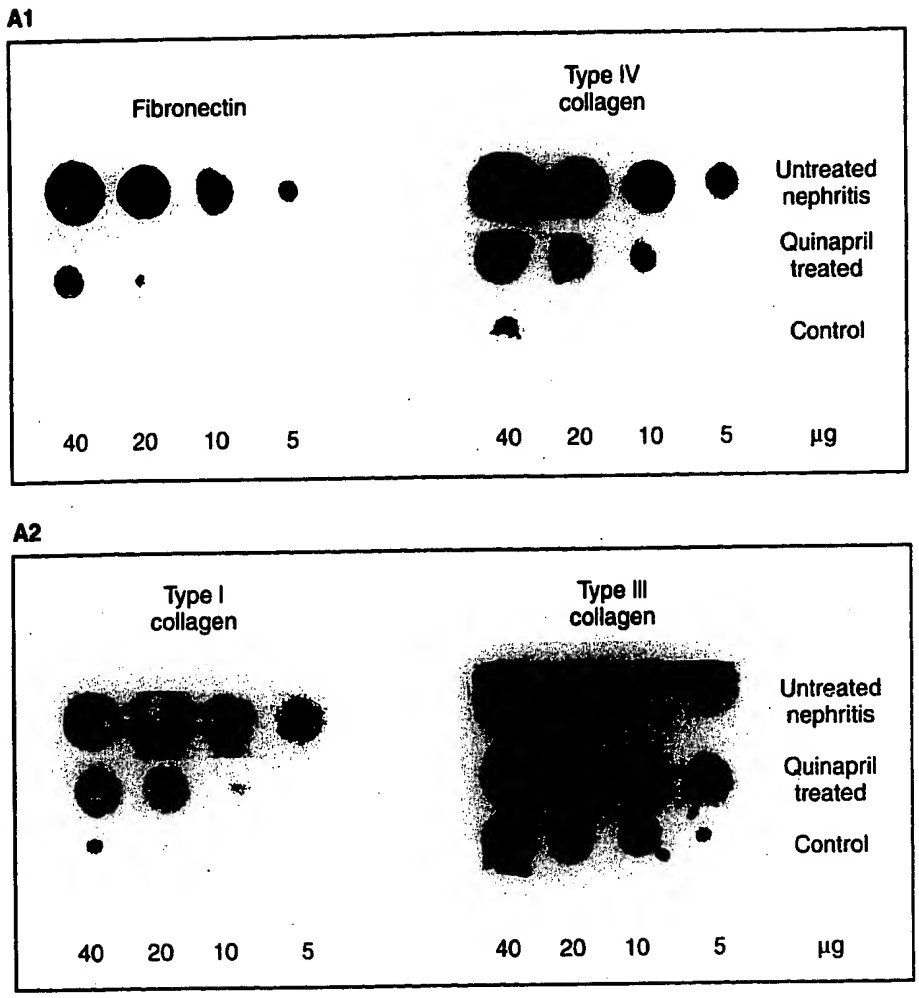

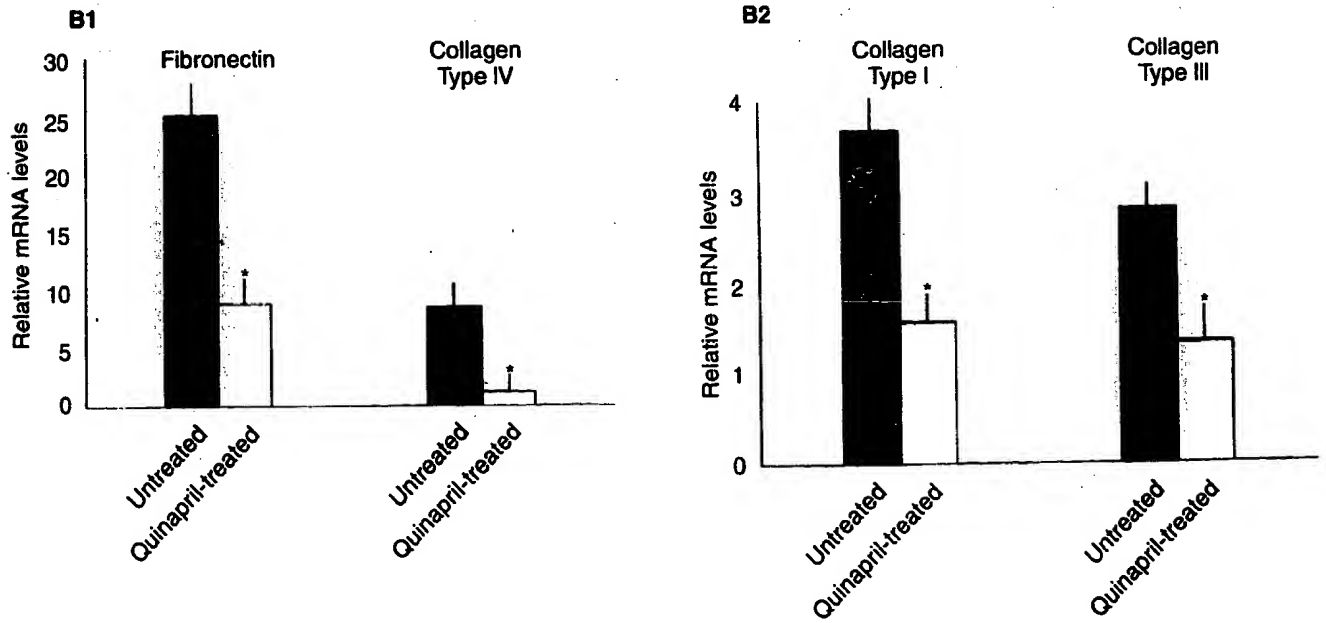

Fig. 10. *Gene expression of matrix proteins in renal cortex.* **(A)** Dot blot analysis. Serial dilutions of RNA from each rat were hybridized with appropriate cDNA probes for fibronectin and type I, III and IV collagens. A representative animal of each group is shown. $N = 4$ to 6 per group. **(B)** Densitometric analysis of dot blot. Values were obtained from the second dot containing 20 µg RNA corrected by the density of 28S and are expressed as N-fold increase over control, mean ± SD of 4 to 6 animals per group. $^*P < 0.05$.

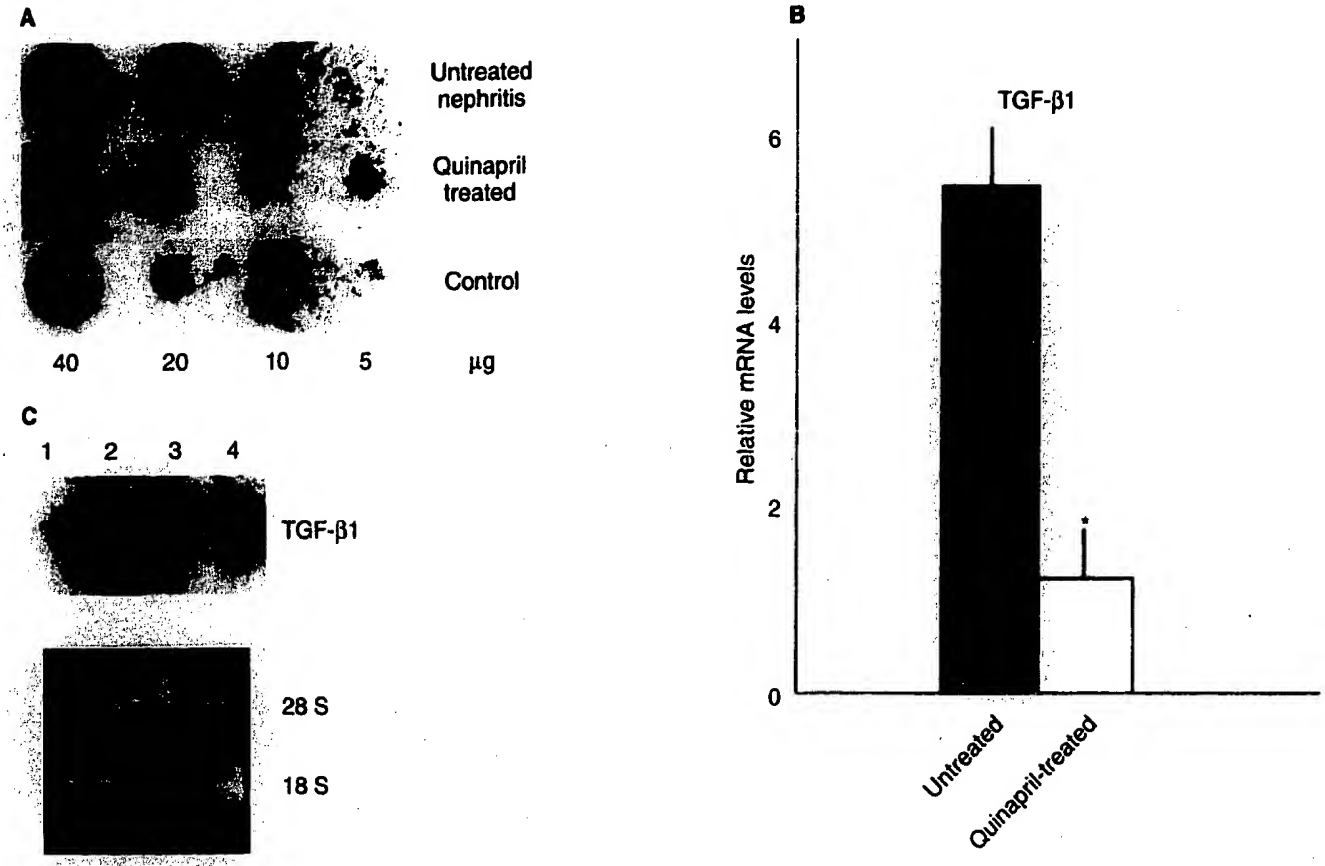

Fig. 11. *Gene expression of TGF-β1 mRNA in renal cortex.* (A) Dot blot analysis. Serial dilutions of RNA from each rat were hybridized with TGF-β1. The Figure shows a representative animal of each group. $N = 4$ to 6 per group. (B) Densitometric analysis of dot blot. Values were obtained from the second dot containing 20 μg RNA corrected by the density of 28S and were expressed as N-fold increase over control. Data are mean ± SD of 4 to 6 animals per group. $^*P < 0.05$. (C) Northern blot. Animals were pooled (4 animals per group), 30 μg of RNA were loaded. Ethidium bromide staining showed the equivalent loading of RNA and its absence of degradation. Lane 1 (healthy controls); Lane 2 (untreated nephritis); Lane 3 (quinapril-treated controls); Lane 4 (quinapril-treated nephritis).

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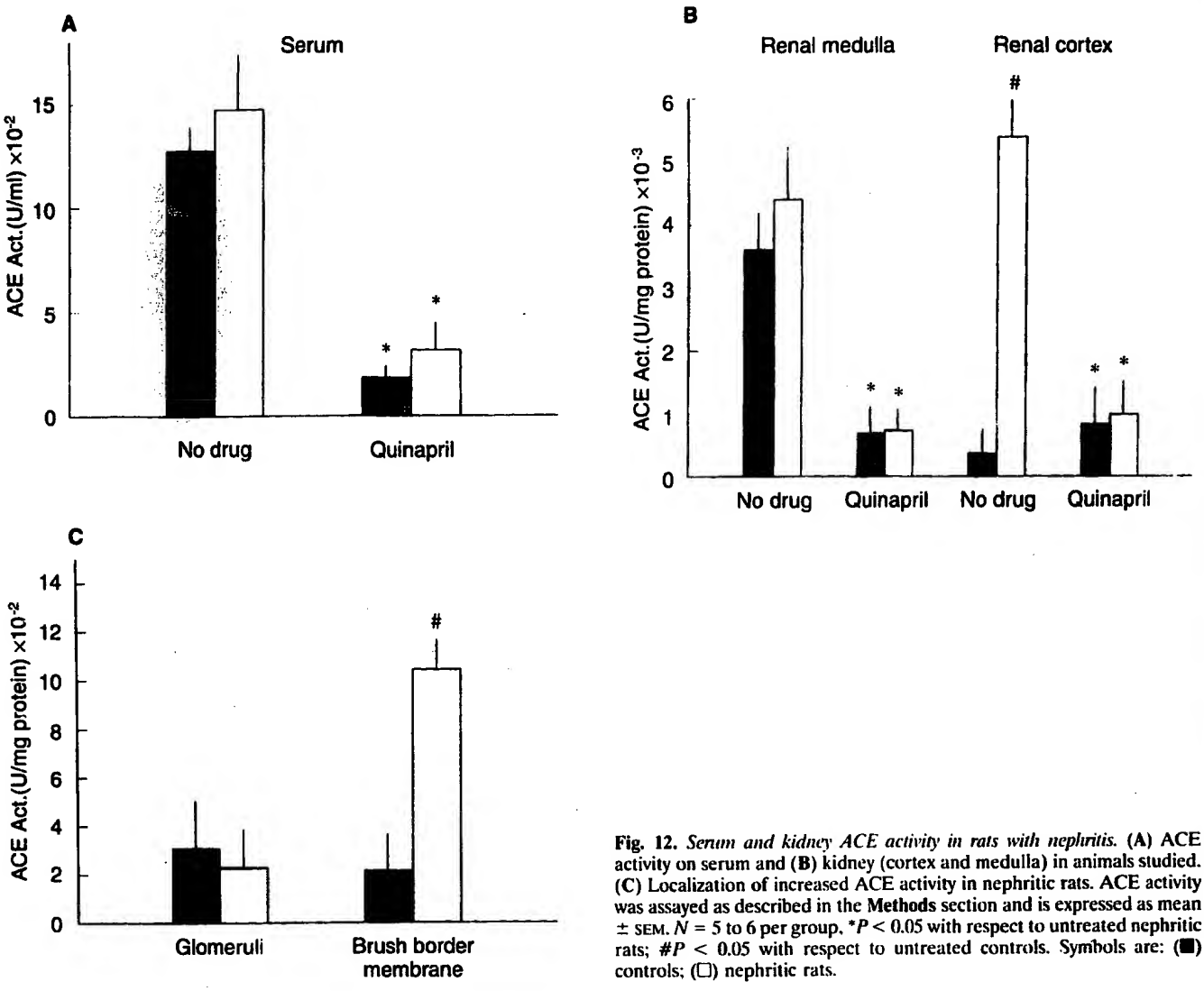

Fig. 12. *Serum and kidney ACE activity in rats with nephritis.* **(A)** ACE activity on serum and **(B)** kidney (cortex and medulla) in animals studied. **(C)** Localization of increased ACE activity in nephritic rats. ACE activity was assayed as described in the **Methods** section and is expressed as mean ± SEM. $N = 5$ to 6 per group, $^*P < 0.05$ with respect to untreated nephritic rats; $\#P < 0.05$ with respect to untreated controls. Symbols are: (■) controls; (□) nephritic rats.

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L6 ANSWER 23 OF 40 CAPLUS COPYRIGHT 2003 ACS
AN 1999:6597 CAPLUS
DN 130:232278
TI Angiotensin converting enzyme inhibition reduces the expression of transforming growth factor-.beta.1 and type IV collagen in diabetic vasculopathy
AU Rumble, Jonathan R.; Gilbert, Richard E.; Cox, Alison; Wu, Leonard; Cooper, Mark E.
CS Department of Medicine, Austin & Repatriation Medical Centre, University of Melbourne, Heidelberg, VIC 3081, Australia
SO Journal of Hypertension (1998), 16(11), 1603-1609
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L6 ANSWER 24 OF 40 MEDLINE DUPLICATE 9
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TI Targeting TGF-beta overexpression in renal disease: maximizing the antifibrotic action of angiotensin II blockade.
AU Peters H; Border W A; Noble N A
CS Division of Nephrology, University of Utah School of Medicine, Salt Lake City, Utah, USA.
NC DK 43609 (NIDDK)
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SO KIDNEY INTERNATIONAL, (1998 Nov) 54 (5) 1570-80.
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GA The Genuine Article (R) Number: YY259
TI Expression of transforming growth factor-beta 1 and type IV collagen in the renal tubulointerstitium in experimental diabetes - Effects of ACE inhibition
AU Gilbert R E (Reprint); Cox A; Wu L L; Allen T J; Hulthen U L; Jerums G; Cooper M E

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Angiotensin converting enzyme inhibition reduces the expression of transforming growth factor-β_1 and type IV collagen in diabetic vasculopathy

Jonathan R. Rumble, Richard E. Gilbert, Alison Cox, Leonard Wu and Mark E. Cooper

Objective **The purpose of this study was to assess the role of transforming growth factor (TGF)-β_1 in the development of diabetes-associated mesenteric vascular hypertrophy and in the antitrophic effect of angiotensin converting enzyme inhibitors.**

Design and methods **Streptozotocin-induced diabetic and control Sprague–Dawley rats were randomly allocated to treatment with the angiotensin converting enzyme inhibitor ramipril or to no treatment and were killed 1 or 3 weeks after the streptozotocin injection. Blood was collected and mesenteric vessels removed. Mesenteric vascular weight was measured and TGF-β_1 and α_1 (type IV) collagen messenger (m)RNA levels were analysed by Northern analysis. Immunohistochemical analyses for TGF-β_1 and type IV collagen were also performed.**

Results **The diabetic rats had increased mesenteric vessel weight at 3 weeks but not at 1 week and a concomitant rise in mesenteric TGF-β_1 and in α_1 (type IV) collagen mRNA levels. Ramipril treatment attenuated mesenteric vessel hypertrophy and prevented the increase in TGF-β_1 and α_1 (type IV) collagen mRNA levels after 3 weeks of diabetes. The immunohistochemical analysis revealed that diabetes was associated with increased TGF-β_1 and type IV collagen protein and extracellular matrix accumulation in mesenteric vessels, and this increase was reduced by ramipril treatment.**

Conclusions **These results support the concept that TGF-β is involved in the changes associated with diabetic vascular disease, and suggest a mechanism by which angiotensin converting enzyme inhibitors exert their antitrophic effects.** ***J Hypertens*** **1998, 16:1603–1609**

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Keywords: mesenteric arteries, transforming growth factor-β, diabetes mellitus, angiotensin II, angiotensin converting enzyme inhibition

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Introduction

Diabetes is associated with microvascular abnormalities which affect various microcirculatory beds, including the kidney and the eye, ultimately leading to nephropathy and retinopathy [1]. Extracellular matrix (ECM) expansion and vascular hypertrophy have been implicated in the pathogenesis of these vascular complications of diabetes [2]. We have reported previously [3,4] that the mesenteric vascular tree undergoes hypertrophy in experimental diabetes, and that these changes are attenuated by angiotensin converting enzyme (ACE) inhibition. This antitrophic effect occurred without effects on food intake, gut weight or glycaemic control [3].

ACE inhibitors have demonstrated antitrophic and antiproliferative effects in nondiabetic models of cardiovascular hypertrophy and neointima formation [5–7]. In experimental diabetes, ACE inhibitors have also been shown to attenuate glomerular hypertrophy [8,9]. In the clinical setting, ACE inhibitors have been shown to promote the preservation of renal function in diabetic patients with nephropathy [10,11].

Transforming growth factor (TGF)-β is a prosclerotic cytokine which exhibits bifunctional growth-promoting effects on cells [12] and is a potent inducer of ECM formation [13]. Diabetic renal disease is associated with cellular

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hypertrophy/hyperplasia and increased ECM deposition [14]. Increased renal TGF-β gene expression and protein levels have been detected in the kidney in experimental and human diabetes [15,16]. Thus, this cytokine has been implicated in the pathogenesis of diabetic nephropathy [17]. Furthermore, in-vitro studies have suggested that angiotensin II stimulates TGF-β expression in vascular smooth muscle cells [18].

In the present study, we assessed the relationship of the expression of TGF-β_1 to the development of mesenteric vascular hypertrophy in experimental diabetes, and the effect of ACE inhibition on this growth factor. Furthermore, the possibility that changes in the gene expression of TGF-β_1 are translated into effects on ECM components was explored by evaluating collagen expression, which has been shown to be dependent on TGF-β [19].

Materials and methods

Male Sprague–Dawley rats aged 8 weeks, weighing between 200 and 250 g, were randomly allocated to treatment with streptozotocin at a dose of 45 mg/kg (diabetic) or to citrate buffer alone (vehicle control). The animals were then further randomized to either no treatment or treatment with the ACE inhibitor ramipril (Hoechst, Frankfurt-am-Main, Germany) in their drinking water (diabetic dose 1.2 mg/l, control 3.6 mg/l), yielding four treatment groups: control, control with ramipril treatment, diabetes and diabetes with ramipril treatment. All rats were given free access to standard chow containing 20% protein (Clark, King & Co, Melbourne, Australia). Only streptozotocin-treated rats with plasma glucose levels of > 15 mmol/l were considered diabetic and included in the study. In this study, streptozotocin administration was associated with 100% of the animals having glucose levels of > 15 mmol/l. The rats were killed after 1 week when mesenteric vascular hypertrophy was not evident, and after 3 weeks when vascular hypertrophy was clearly apparent.

Immediately before they were killed, the rats were weighed and systolic blood pressure was determined by tail-cuff plethysmography [20]. They were killed by decapitation and blood was collected for the determination of plasma glucose by the glucose oxidase technique [21]. The mesenteric vessels were then removed and stripped of surrounding fat, connective tissue and veins to yield the superior mesenteric arterial tree, as described previously [3]. The vessels were weighed, snap-frozen in liquid nitrogen and subsequently stored at –80°C.

Northern analysis

Mesenteric arteries stored at –80°C were homogenized (Ultra-Turrax, Janke and Kunkel, Staufen, Germany) and total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method [22]. RNA purity and concentration were determined spectrophotometrically. Twenty-microgram samples were denatured and electrophoresed through 0.8% agarose formaldehyde gels. RNA integrity was verified by examination of the 28S and 18S ribosomal (r)RNA bands of ethidium bromide-stained material under ultraviolet light. RNA was then transferred onto nylon filters (Hybond-N, Amersham, UK) by capillary action and fixed by ultraviolet irradiation.

Filters were hybridized with a 985 bp complementary (c)DNA probe coding for rat TGF-β_1 (gift from S.W. Qian, NIH, Bethesda, Maryland, USA) and a 1.8 kb cDNA probe coding for mouse α_1 (type IV) collagen (gift from R. Timpl, Max Plank Institute, Martinsried, Germany). The probes were labelled with α-^{32}P deoxy (d)CTP (DuPont, Boston, Massachussetts, USA) by random primed DNA synthesis (Boehringer Mannheim, Mannheim, Germany). For TGF-β_1, hybridization was performed at 65°C for 16–20 h in a solution containing 1% bovine serum albumin, 7% sodium dodecyl sulphate, 0.5 mol/l $NaHPO_4$ and 1 mmol/l ethylenediaminetetraacetic acid (EDTA) followed by three washes at 65°C for 15 min in 1% sodium dodecyl sulphate, 40 mmol/l $NaHPO_4$ and 1 mmol/l EDTA. The filters for α_1 (type IV) collagen were hybridized at 42°C for 24 h in 50% formamide, 45 mmol/l Na_2HPO_4, 5 × Denhardt's solution, 0.5% sodium dodecyl sulphate and sonicated salmon sperm DNA. The filters were then washed in solutions of decreasing ionic strength and increasing temperature. The final stringency was 0.1 × standard saline citrate with 0.1% sodium dodecyl sulphate for 20 min at 42°C. The intensity of hybridization was quantified using a phosphorescent imager (Fujix BASS 3000, Fuji Photo Film Co., Ltd, Tokyo, Japan). All results were corrected for differences in RNA loading and transfer by rehybridization with an oligonucleotide probe for 18S rRNA end labelled with α-^{32}P dCTP by terminal transferase (Boehringer Mannheim). Results were expressed as the ratio of the image intensity of TGF-β_1 or α_1 (type IV) collagen to 18S, relative to control vessels which were arbitrarily assigned a value of 1.

Immunohistochemical analysis

TGF-β_1 and type IV collagen in blood vessels, obtained from animals 3 weeks after the induction of diabetes, were evaluated immunohistochemically using a polyclonal rabbit anti-TGF-β_1 antibody (R and D Systems, Minneapolis, Minnesota, USA) and a polyclonal goat antibovine/antihuman α_1 (type IV) antibody (Southern Biotechnology, Birmingham, Alabama, USA). Tissues were fixed in methyl Carnoy's solution and embedded in paraffin. Four-micron sections were then cut and immunohistochemistry performed using a standard immunohistochemical technique, as described previously [23].

To further evaluate the degree of matrix accumulation in the medial layer of these vessels, sections were stained

with Masson's trichrome [24,25]. Four-micron sections were rehydrated and immersed in potassium dichromate–alcoholic hydrochloric acid for 30 min, and then rinsed in water. The sections were then stained with Celestine Blue–haematoxylin and rinsed again in water. They were subsequently treated with 1% Brilliant Crocein in 1% phosphotungstic acid for 5 min and rinsed in 1% phosphotungstic acid. The final stain was performed by immersing the slides in 0.5% Light Green in 1% acetic acid for 10 min and then washing them with 1% aqueous acetic acid. Finally the sections were dehydrated, cleared and mounted. With this method, nuclei are stained black, smooth muscle is stained red and collagen is stained blue. The blue staining that represents collagen was quantified by a videoimaging system (Video Pro 32, Leading Edge, Bedford Park, South Australia, Australia), connected to a Zeiss Asiophot microscope (Oberkocken, Germany). With this device, the outline of the medial layer was defined by interactive tracing. The area of the media stained blue was then determined using a computerized image analysis system (Analytical Imaging Station, Imaging Research Inc., St Catherine's, Ontario, Canada). The degree of collagen deposition was calculated as the quotient of the area stained blue and the total medial area.

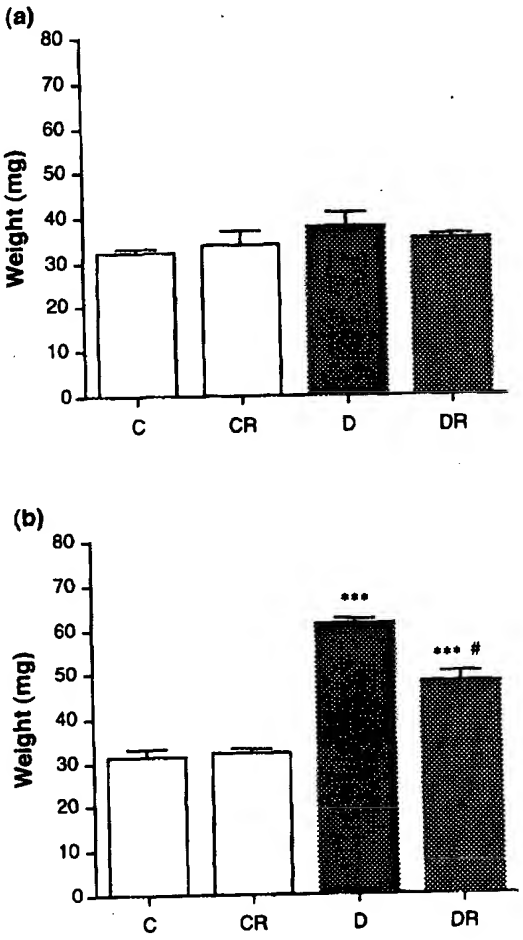
Statistics

Values are shown as means ± SEM. Data were analysed by analysis of variance using the StatView II programme (Abacus Concepts Inc., Berkeley, California, USA) on a Macintosh computer (Apple, Cupertino, California, USA). Comparisons between group means were analysed by Fisher's least significant difference method [26]. $P < 0.05$ was considered statistically significant.

Results

Results for body weight, plasma glucose and blood pressure are shown in Table 1. Diabetes was associated with reduced weight gain and increased plasma glucose levels which were not influenced by ramipril treatment. Systolic blood pressure was significantly higher in the diabetic groups on day 7 and after 3 weeks, compared with controls, and the increase was attenuated by ramipril treatment. Ramipril treatment decreased blood pressure

Fig. 1



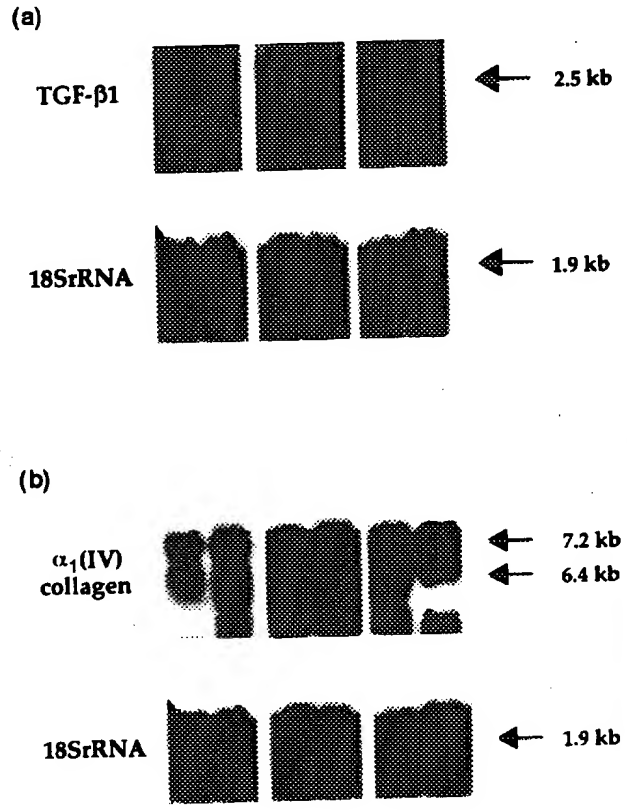
Mesenteric vessel weight (means ± SEM) on day 7 (panel a) and after 3 weeks (panel b). C, control; R, ramipril; D, diabetic. ***$P < 0.001$ versus control; #$P < 0.001$ versus diabetic rats.

Table 1 **Clinical characteristics of study rats**

	n	Body weight (g)	Plasma glucose (mmol/l)	Blood pressure (mmHg)
Rats killed at 7 days				
Control	10	303 ± 7	7.2 ± 0.1	131 ± 3
Control + R	6	300 ± 10	6.5 ± 0.3	123 ± 4
Diabetic	6	233 ± 15***	24.3 ± 1.8***	154 ± 7*
Diabetic + R	12	252 ± 13**	26.3 ± 1.7***	131 ± 8†
Rats killed at 3 weeks				
Control	9	315 ± 10	6.6 ± 0.2	133 ± 5
Control + R	9	289 ± 6	6.5 ± 0.2	120 ± 3*
Diabetic	12	248 ± 8***	27.6 ± 0.7***	163 ± 3***
Diabetic + R	12	235 ± 12***	29.2 ± 2.2***	136 ± 3††

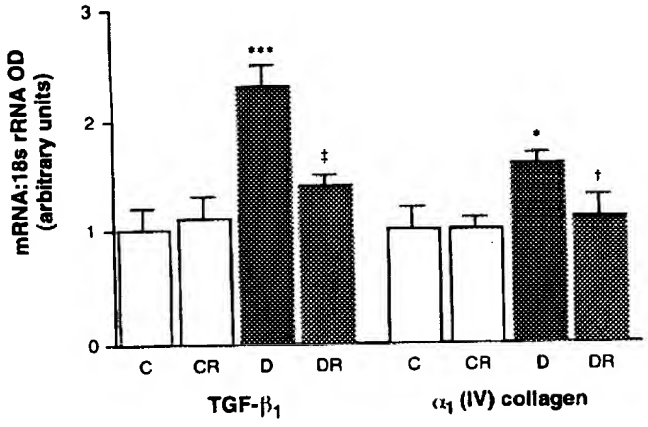
Values are means ± SEM. R, ramipril. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, versus control; †$P < 0.05$, ††$P < 0.001$, versus diabetic rats.

Fig. 2



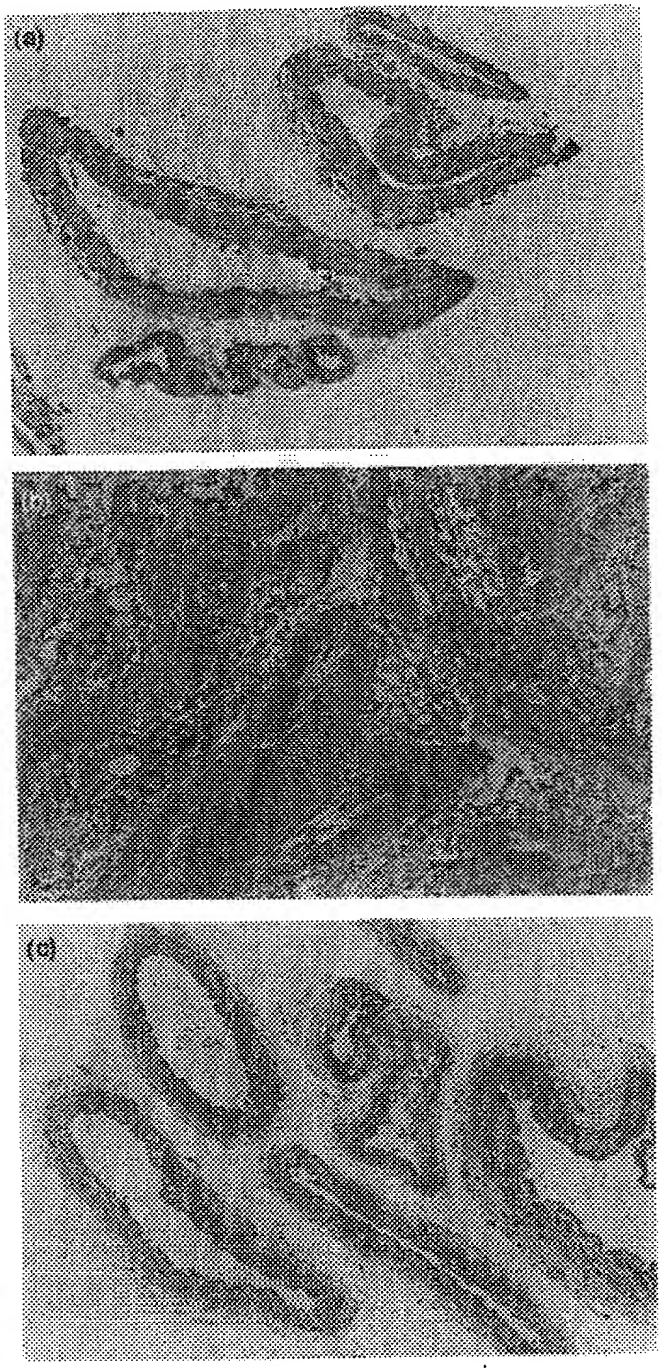
Northern blots of mesenteric vessel messenger ribosomal (r)RNA for transforming growth factor (TGF)-β_1 (a) and type IV collagen (b) in control, diabetic and diabetic + ramipril-treated rats (left to right, respectively) after 3 weeks.

Fig. 3



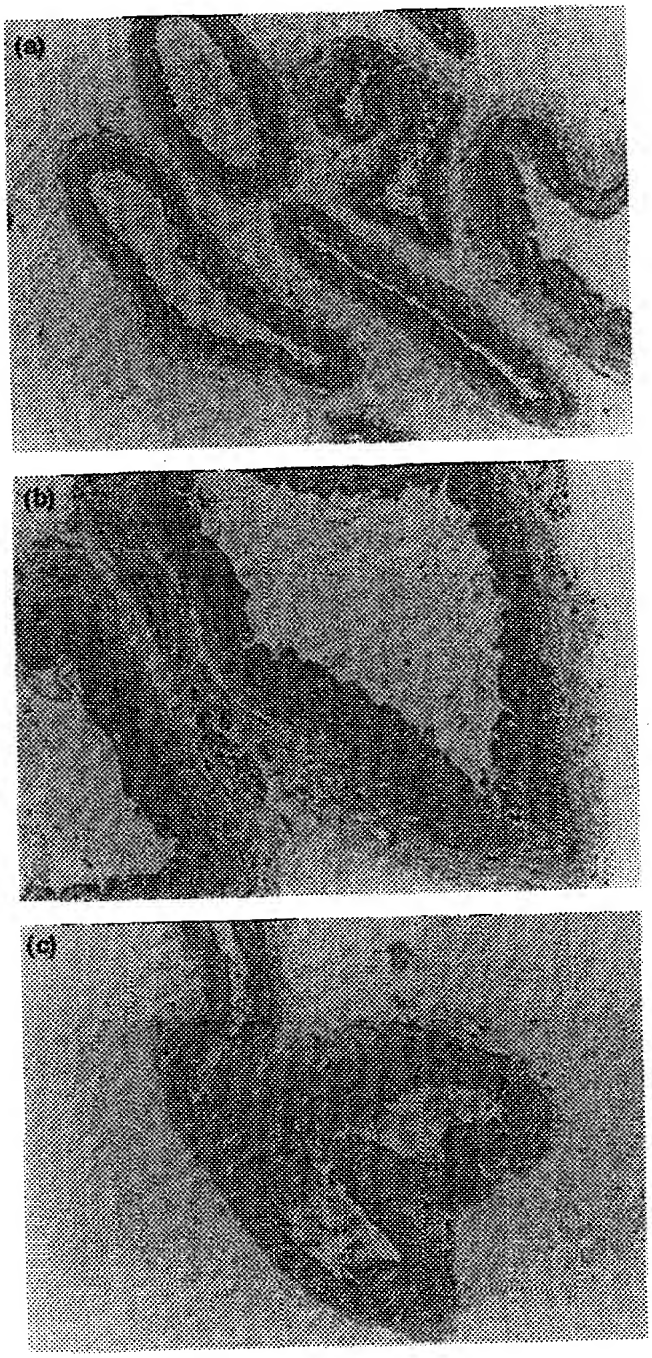
Mesenteric transforming growth factor (TGF)-β_1 and α_1 (type IV) collagen messenger (m)RNA in control (C), control + ramipril-treated (CR), diabetic (D) and diabetic + ramipril-treated (R) rats at 3 weeks. Data are means ± SEM of the ratio of optical density (OD) of specific mRNA to that of 18S ribosomal (r)RNA, relative to that of control rats (designated an arbitrary value of 1). $^*P < 0.05$, $^{***}P < 0.001$, versus control; $^{\ddagger}P < 0.05$, $^{\dagger}P < 0.01$, versus diabetic rats.

Fig. 4



Transforming growth factor-β_1 in media stained with antibody to this factor in (a) control, (b) diabetic and (c) diabetic + ramipril-treated rats (magnification ×200).

Fig. 5



Type IV collagen in media stained with antibody to α_1 (type IV) collagen in (A) control, (B) diabetic and (C) diabetic + ramipril-treated rats (magnification ×200).

in nondiabetic rats, reaching statistical significance only at the 3-week time point. After 1 week of diabetes, there was no increase in mesenteric vascular weight (Fig. 1a). The mesenteric vessel weight was increased in untreated diabetic rats, compared with control animals, at 3 weeks (Fig. 1b). Ramipril treatment attenuated the increase in mesenteric vascular weight in the diabetic animals.

Northern analysis

Mesenteric vessel TGF-β_1 messenger (m)RNA was at least doubled in the diabetic rats by weeks 1 and 3 compared with their nondiabetic counterparts. Ramipril prevented this overexpression of TGF-β_1 after 3 weeks (Figs 2a, 3), but not after 1 week (control, 1.0 ± 0.1; control + ramipril, 1.3 ± 0.1; diabetic, 4.2 ± 0.6; diabetic + ramipril, 5.5 ± 0.9 arbitrary units; $P < 0.01$, diabetic groups versus controls). Similarly, α_1 (type IV) collagen gene expression was increased in the diabetic rats at both time points and the increase was attenuated by ramipril treatment at the 3-week time point (Fig. 2b, 3), but not at 1 week (control, 1.0 ± 0.1; control + ramipril, 1.0 ± 0.1; diabetic, 1.5 ± 0.2; diabetic + ramipril, 1.8 ± 0.4 arbitrary units; $P < 0.05$ diabetic groups versus controls). Ramipril treatment had no effect on TGF-β_1 or α_1 (type IV) collagen mRNA levels in control rats.

Immunohistochemical analysis

Mesenteric vessels from control rats showed minimal evidence of TGF-β_1 protein (Fig. 4a). By contrast, after 3 weeks of experimental diabetes, there was widespread TGF-β_1 protein, particularly in the media but also in cells of the adventitial layer (Fig. 4b). Ramipril-treated rats showed a reduction in TGF-β_1 (Fig. 4c).

To determine whether these effects on TGF-β_1 were translated into effects on ECM, we performed an immunohistochemical analysis of type IV collagen. Diabetes was associated with an increase in type IV collagen, especially in the medial layer, but also in the adventitia (Fig. 5b). The expression of this matrix protein was decreased in the ramipril-treated diabetic group (Fig. 5c).

To further assess the degree of ECM accumulation in the vessel wall, the proportion of collagen in the medial layer was determined using a Trichrome stain [24]. Diabetes was associated with increased collagen deposition (control, $n = 5$, $5.2 \pm 2.2\%$; diabetic, $n = 6$, $15.5 \pm 2.8\%$; $P < 0.05$). Ramipril reduced but did not normalize the proportion of collagen in the vessel wall (diabetic + ramipril, $n = 5$, $8.6 \pm 1.7\%$).

Discussion

In the present study, as reported previously [3], experimental diabetes was associated with mesenteric vascular hypertrophy after 3 weeks. This increase in mesenteric vascular growth was accompanied by a concomitant increase in gene expression of the prosclerotic cytokine

TGF-β_1 and also of the ECM component α_1 (type IV) collagen in these vessels. Treatment of the diabetic animals with the ACE inhibitor ramipril attenuated vascular hypertrophy and prevented the overexpression of TGF-β_1 and α_1 (type IV) collagen mRNA and protein in these mesenteric vessels.

Angiotensin II is integrally involved in cardiovascular homeostasis, particularly in the regulation of blood pressure and blood volume [27]. However, other effects of angiotensin II, such as its growth-promoting actions, have been implicated in the pathogenesis of disease states such as hypertension, atherosclerosis and diabetes [28]. In cell culture, angiotensin II has been shown to exert growth-promoting effects on vascular smooth muscle cells [18,29], and to stimulate ECM production by these cells [30]. *In vivo*, angiotensin II has been shown to stimulate vascular smooth muscle cell proliferation in normal and injured vessels [31], and increased local production of angiotensin II caused vascular hypertrophy of the carotid arteries in rats [32].

TGF-β is a proselerotic cytokine which has been shown to cause both hypertrophy and hyperplasia of vascular smooth muscle cells [18]. As well as its growth-promoting effects on cells, TGF-β has potent effects on ECM production and accumulation [13]; it has also been shown to mediate angiotensin II-induced effects on cellular growth and ECM production *in vitro* [18,33]. However, the possibility that angiotensin II mediates its growth-promoting effects *in vivo* via TGF-β has not been as clearly demonstrated.

A previous study in our laboratory showed that mesenteric vessel ACE was increased in diabetic rats [3]. Anderson *et al.* [34] have shown that glomerular ACE is increased in experimental diabetes. Thus, we speculate that in these sites in diabetes, the increase in tissue ACE leads to increased local angiotensin II production which leads to increased TGF-β gene expression. Therefore, the antitrophic effects of ACE inhibitors may be mediated by inhibiting angiotensin II-induced TGF-β gene expression. The present study provides in-vivo evidence suggesting a link between angiotensin II and TGF-β in blood vessels, particularly in the presence of diabetes. Recently, in a long-term study of diabetic rats [16], our group has demonstrated that a similar phenomenon occurs in the kidney, with ramipril attenuating the expression of renal TGF-β and type IV collagen.

Of particular relevance in experimental diabetes is the in-vitro observation that glucose as well as angiotensin II has been shown to stimulate cell growth and ECM synthesis via increased TGF-β production [19]. Similarly, advanced glycation end-products, which are generated as a result of an interaction between long-lived proteins and chronic hyperglycaemia [35], have been shown to increase gene expression of TGF-β and type IV collagen in the glomeruli of rats [36]. No studies have, as yet, suggested a direct link between these advanced glycosylation end-products and the renin–angiotensin system. Therefore, it has been postulated that high glucose levels, advanced glycation end-products and increased angiotensin II production all contribute to the overexpression of TGF-β in diabetes.

Further evidence implicating TGF-β in the vascular hypertrophy associated with diabetes has been obtained from a recent study in our laboratory using an inhibitor of advanced glycation, aminoguanidine, in diabetic rats [25]. This agent prevented mesenteric vascular hypertrophy and was associated with suppression of TGF-β_1 mRNA levels. Since TGF-β is initially synthesized in a latent form and then converted to an active form, an increase in TGF-β mRNA does not itself represent increased TGF-β action. The concomitant increase in collagen deposition is consistent with evidence of the proselerotic action of TGF-β. Previous work in diabetes has shown a relationship between TGF-β expression and bioactivity [37]. In addition, our group has demonstrated that the increase in TGF-β gene expression in diabetic vessels is associated with an increase in expression of β-inducible gene h3 (βig-h3), a matrix protein that is selectively induced by TGF-β [25]. Nevertheless, for further exploration of the role of TGF-β in diabetic vascular injury, experiments are needed that measure the production of the active form of TGF-β, assess the status of TGF-β receptors and postreceptor events, and use agents that can block specifically TGF-β action.

ACE inhibitors act not only to decrease angiotensin II production but also to decrease the degradation of kinins [38]. Previous studies in our laboratory have indicated that this effect of ACE inhibitors is an important mechanism in their antitrophic effects in experimental diabetes [39]. Thus the ACE inhibitor action that induces an increase in bradykinin and nitric oxide levels may also be involved in the reduction of TGF-β gene expression. Nevertheless, the capacity of angiotensin II antagonists to prevent mesenteric vascular hypertrophy as effectively as ACE inhibitors [39] strongly implicates angiotensin II in the genesis of diabetes-associated microvascular injury. A similar predominance of angiotensin II versus kinin-dependent pathways in mediating the renoprotective properties of ACE inhibitors has recently been reported by our group [40].

In summary, our results in the present study support the concept that the antitrophic effect of ACE inhibitors in experimental diabetes may involve the attenuation of TGF-β action, presumably via suppression of TGF-β gene expression.

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AU Lyons J.G.; Birkedal-Hansen B.; Pierson M.C.; Whitelock J.M.; Birkedal-Hansen H.
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L9 ANSWER 23 OF 37 MEDLINE DUPLICATE 10
AN 96130535 MEDLINE
DN 96130535 PubMed ID: 8544402
TI Induction of plasminogen activator inhibitor type 1 in murine lupus-like glomerulonephritis.
AU Moll S; Menoud P A; Fulpius T; Pastore Y; Takahashi S; Fossati L; Vassalli J D; Sappino A P; Schifferli J A; Izui S
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SO KIDNEY INTERNATIONAL, (1995 Nov) 48 (5) 1459-68. Journal code: 0323470. ISSN: 0085-2538.
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L9 ANSWER 20 OF 37 MEDLINE DUPLICATE 8
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DN 97081970 PubMed ID: 8923213
TI Increased expression of extracellular matrix proteins and decreased expression of matrix proteases after serial passage of glomerular mesangial cells.
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Increased expression of extracellular matrix proteins and decreased expression of matrix proteases after serial passage of glomerular mesangial cells

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SUMMARY

The cellular events causing pathological extracellular matrix (ECM) accumulation in vivo are not well understood. Prolonged serial passage of several cell types in culture leads to increased production of extracellular matrix (ECM) proteins, but the mechanism for these putative fibrotic changes is not known. Here, human fetal glomerular mesangial cells were subjected to serial passage (P) in culture and the expression of ECM proteins, proteases and protease inhibitors was comprehensively evaluated. From P11 through P14, a series of phenotypic changes occurred. Steady-state expression of mRNA for α1 chains of type III and type IV (but not type I) collagen, and for laminin β1 and γ1, increased 2- to 8-fold, while expression of mRNA for interstitial collagenase (MMP-1) and gelatinase A (MMP-2) virtually ceased. Expression of tissue-type plasminogen activator (tPA) mRNA also decreased markedly. Expression of mRNA for the tissue inhibitor of metalloproteinases (TIMP)-1, and of the smaller of two mRNA species for the PA inhibitor PAI-1, ceased by P14. There was a switch in expression of the two species of TIMP-2 mRNA: whereas the ratio of signal intensity comparing the 3.5 kb mRNA species to the 1.0 kb species was 5:1 up to P11, it was reversed (1:5) at P14 and later. Serial passage also led to changes in protein expression, with increased type IV collagen and laminin, but decreased interstitial collagenase and gelatinase A. The cells showed a progressive increase in staining for type IV collagen. These findings define the appearance of a matrix-accumulating phenotype in later-passage mesangial cells.

Matrix expansion in vivo has been associated with increased transforming growth factor (TGF)-β synthesis; the cells were found to show at least 5-fold increased expression of TGF-β1 mRNA from P8 to P16. However, treatment of P9 or P10 cells with graded doses of TGF-β1 increased expression of both collagen IV and gelatinase A mRNA and did not alter the ratio of signal intensity for TIMP-2 mRNA species. Thus, assumption of a matrix-accumulating phenotype by these cultured fetal glomerular mesangial cells is not accelerated by exogenous TGF-β. These data describe an in vitro model of mesangial cell matrix turnover in which matrix accumulation could result from a concerted increase in ECM synthesis and decrease in ECM degradation.

Key words: Glomerulosclerosis, Mesangial cell, Collagen, Extracellular matrix, Extracellular matrix protease

INTRODUCTION

Under certain pathological conditions, the accumulation of excess extracellular matrix (ECM) in tissues leads to dysfunction. This matrix accumulation, part of the process of sclerosis, may cause thickening of basement membranes, disruption of normal cell-cell interactions, and loss of tissue compliance or elasticity in such conditions as atherosclerosis of the blood vessels (Raines and Ross, 1991), pulmonary fibrosis (McGowan, 1992), liver cirrhosis (Rojkind, 1991), and destruction of the glomerulus that filters blood in the kidney. Glomerulosclerosis involves glomerular structural collapse and ECM accumulation (Thoenes and Rumpelt, 1977). It may be a primary manifestation of glomerular disease, but it also represents a common series of events by which function is lost in a variety of kidney diseases (Schnaper, 1996). Although several factors have been implicated in causing glomerulosclerosis, the underlying cellular mechanisms which lead to matrix accumulation are less well understood. Most studies of glomerular scarring have emphasized the role of increased ECM synthesis in matrix expansion (Adler et al., 1986; Brazy et al., 1991; Kopp et al., 1992). However, it is likely that the net rate of matrix turnover is more important than the absolute rate of ECM protein synthesis alone. Thus, increasing attention

has been focused on the rate at which extracellular matrix degradation occurs, since the balance between synthesis and degradation of ECM components determines the accumulation or loss of matrix (Couchman et al., 1994).

The two most abundant structural proteins in the glomerular ECM are type IV collagen and laminin. These proteins are the major matrix components that accumulate in progressive glomerular disease. In addition, type I and type III collagen may accumulate in the interstitium (Jones et al., 1991) or the glomerulus (Striker et al., 1984). Two major proteolytic pathways are important in degradation of these molecules. In one, the matrix metalloproteinases (MMPs), a family of neutral zinc proteases, degrade the different ECM components with varying specificities (Birkedal-Hansen, 1993). The most well-studied of these are the collagenases and gelatinases that degrade interstitial/fibrillar (types I and III) and basement membrane (type IV) collagens, respectively. MMP activity is blocked by a group of endogenous inhibitory proteins termed tissue inhibitors of metalloproteinases (TIMPs) (Liotta et al., 1991). The second proteolytic pathway important in ECM degradation includes both the plasminogen activators that convert plasminogen to plasmin, and plasmin itself. These serine proteases are important in laminin degradation (Liotta et al., 1981). Plasminogen activators are blocked by the plasminogen activator inhibitors (PAIs) (Collen, 1980). Together, these two proteolytic systems serve as the major pathways of degradation affecting matrix turnover. Both have been implicated in matrix degradation by glomerular cells (Baricos et al., 1995). Changes in the amount of ECM proteases produced in the tissue, in their activity, or in the ratios between proteases and their inhibitors, should have a significant effect on the net accretion of ECM proteins in the kidney. Support for this hypothesis is found in the observation that, in rats induced to develop glomerulosclerosis by administration of puromycin aminonucleoside, progressive interstitial fibrosis (Jones et al., 1991) and glomerular sclerosis (Nakamura et al., 1994) are accompanied by shifts in the balance between ECM protein synthesis and degradation that would favor matrix accumulation; amelioration of the disease progression is associated with a reversal of these changes (Nakamura et al., 1994).

The cellular mechanisms by which the balance in matrix turnover is altered in glomerulosclerosis is not known. We propose that specific alterations in cell structure and function are associated with matrix accumulation. Thus, by determining characteristics of matrix-accumulating cells, it may be possible to identify cellular events that are potentially related to the pathogenesis of glomerulosclerosis. Mesangial cells occupy a central position in the renal glomerulus at the location where matrix accumulation is first noted in classical focal glomerulosclerosis (Schnaper, 1996). In the studies described here, human fetal mesangial cells were subjected to a prolonged series of passages in culture. This manipulation led to assumption of a matrix-accumulating phenotype, associated with increased expression of ECM components but decreased expression of several ECM proteases at the level of both mRNA and protein. In addition, expression of different mRNA species for two protease inhibitors varied with serial passage. These changes are not caused by exogenous transforming growth factor (TGF)-β1, a known stimulus of the sclerotic process. Instead, this phenotypic change by the cultured cells appears to result from another effect, yet to be determined. This model should prove useful for investigating the cellular mechanisms by which changes that contribute to matrix accumulation occur in vivo.

MATERIALS AND METHODS

Unless otherwise stated, all materials used are reagent grade and were purchased from commercial sources. Active, human recombinant TGF-β1 produced in CHO cells was purchased from R&D systems, Minneapolis, MN.

Culture and passage of the glomerular mesangial cells

Mesangial cells were isolated by differential sieving of minced glomerular tissue (Harper et al., 1984) obtained from a fetus after an elective therapeutic abortion at 14-15 weeks' gestation. The cells in this isolate, termed FMC2, were confirmed to be mesangial by morphological criteria, by the presence of abundant actin microfilaments, and by the absence of staining for cytokeratin and factor VIII-related antigen (Weeks et al., 1991). Confluent cultures of cells were split 1:5 in DMEM/Ham's F12 medium supplemented with 20% heat-inactivated fetal calf serum (Hyclone; Logan, UT), glutamine, penicillin/streptomycin, sodium pyruvate, Hepes buffer, and 8 μg/ml insulin (Life Technologies, Gaithersburg, MD). Aliquots of each passage from P7 to P25 were frozen in 20% serum, 10% DMSO and preserved in liquid nitrogen for future use so that different passages of the cells could be assayed at the same time under identical circumstances. The cells were determined to be mycoplasma-free by Hoechst staining. Vials of cells frozen at different passages were thawed quickly and cultured, usually through one additional passage, before they were evaluated.

Isolation of mesangial cell RNA

Cells were lysed in 4 M guanidine isothiocyanate, 25 mM sodium acetate, and 100 mM β-mercaptoethanol (Sambrook et al., 1989) and removed from the plate by scraping. After centrifugation over a $CsCl_2$ cushion (Glisin et al., 1974) overnight at 32,000 rpm, the RNA pellet was resuspended in 0.3 M sodium acetate, phenol/chloroform extracted, and precipitated with chilled, absolute ethanol. After centrifugation, the pellet was washed with 70% ethanol, dried, and resuspended in DEPC-treated water before quantification by absorption at 260 nm.

Northern analysis

Total cellular RNA (10 μg) was subjected to denaturing (formaldehyde) gel electrophoresis through 1% agarose in Mops buffer before overnight transfer to nylon membrane by capillary action. The RNA was crosslinked to the membrane by ultraviolet irradiation. ^{32}P-labeled cDNAs were hybridized with the blots in QuickHyb (Stratagene) at 65°C, for 1 hour. The blots were washed at serially increasing stringency, dried, and exposed to pre-flashed X-ray film at –70°C for the purposes of photography. Signal intensity was quantified using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Studies of mRNA expression were confirmed by evaluation of at least three different preparations of RNA from cells harvested at different times; in some instances, RNA from the same preparations was subjected to electrophoresis and transfer more than once, with similar results on separately hybridized blots.

cDNA probes used in hybridization

cDNAs for human α1(I), α1(III) and α1(IV) collagen chains, and for laminin α1 (representing the carboxy-terminal end of the EHS laminin α chain), β1, and γ1 chains, were obtained from L. Bruggeman and Y. Yamada, National Institute of Dental Research, NIH. The probes for gelatinase A (MMP-2) (Collier et al., 1988) and gelatinase B (MMP-9) (Wilhelm et al., 1989) were obtained from G. Goldberg,

Washington University, St Louis, MO. The TIMP-1 cDNA (Carmichael et al., 1986) was obtained from D. Carmichael, Synergen, Boulder, CO; TIMP-3 cDNA was obtained from D. Edwards, University of Calgary. The TIMP-2 (Stetler-Stevenson et al., 1990) and interstitial collagenase (Brown et al., 1990) cDNAs were prepared as described. Probes for tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) were kindly provided by D. Dichek, NCI (Dichek et al., 1989). The cDNA for PAI-1 (Feinberg et al., 1989) was provided by E. S. Barnathan, University of Pennsylvania. The PAI-2 cDNA (Ye et al., 1987) was obtained from the American Type Culture Collection. Blots were hybridized with cDNA for 28 S (H. Sage, University of Washington, Seattle WA) or 18 S (D. Edwards) ribosomal RNA to control for loading.

Immunoblot analysis

Cells from different passages were plated at identical density in 150-mm culture dishes. Three days later, before the cells were confluent, the medium was replaced with serum-free medium. After 18 hours, the supernatant fluid was harvested and subjected to SDS-PAGE in a 10% gel under reducing conditions before electrophoretic transfer to Immobilon-P membranes (Millipore). The membranes were dried, spotted with positive controls, and blocked with BSA. They were then developed with a 1:50 dilution of sheep anti-mouse type I collagen or 1:100 dilution of rabbit anti-mouse type IV collagen or rabbit anti-mouse laminin. These antibodies were prepared by immunizing the respective animals against mouse skin type I collagen or against collagen IV (Kleinman et al., 1982) or laminin (Timpl et al., 1979) purified from the Engelbreth-Holm Swarm tumor; they have been shown to cross-react with human proteins. After washing, the blots were incubated with 1:500 antiserum against the primary antibody, conjugated to horseradish peroxidase. Finally, the signal was developed with 4-chloronaphthol/H_2O_2.

Indirect immunofluorescence for type IV collagen

Cells were plated at identical densities (6,000 per well) in an eight-well chamber slide (LabTek) for three days prior to fixation with 3.7% formaldehyde in PBS followed by permeabilization with Triton X-100 in PBS, and then stained with rabbit anti-collagen IV antiserum (1:100) followed by goat anti-rabit IgG-FITC (Vector), 1:100. Control wells were incubated with preimmune rabbit serum. Photomicrographs were taken using a Nikon Optiphot microscope and UFX-DX photographic system with identical times for all exposures.

Zymogram analysis

Serum-free, mesangial cell-conditioned media were evaluated for gelatinase activity as described previously (Schnaper et al., 1993). Fresh or frozen cell supernates were subjected to electrophoresis under non-denaturing conditions through a 10% SDS-polyacrylamide gel containing 1 mg/ml gelatin. The gel was then incubated twice in 2.5% Triton X-100 for 30 minutes each time, and equilibrated in 10 mM Tris, pH 8, before incubation in 50 mM Tris containing 5 mM $CaCl_2$ and 1 μM $ZnCl_2$ for 16 hours at 37°C (Birkedal-Hansen and Taylor, 1982), and then stained with Coomassie blue. Bands of protein with gelatinolytic activity appear as clear zones in the surrounding blue background.

RESULTS

Expression of mRNA for collagens, collagenases and TIMPs

FMC2 cells, thawed at passages 7, 10, 13 and 15, were cultured for one additional passage before total cellular RNA was harvested and subjected to electrophoresis. The blot resulting after northern transfer was hybridized with cDNAs for the α1 chains of types I, III and IV collagen. There was no significant change in the total signal for mRNA for α1(I) collagen (Fig. 1). There was, however, a difference in the pattern of expression observed, with decreased expression of the larger of the two species that were detected. A pronounced change with serial passage was seen when the same blot was stripped and re-probed for expression of both α1(III) and α1(IV). These mRNAs showed a marked increase in steady-state expression. There was a 4-fold increase in signal intensity for α1(III) from P11 to P14 and an additional doubling of intensity at P16 (corrected for loading). Similarly, there was a 2-fold increase in intensity of α1(IV) signal from P11 to P14 and further doubling to P16.

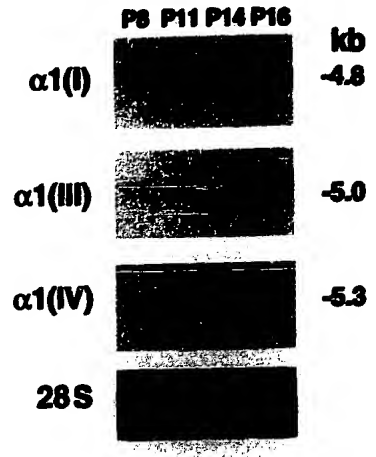

Fig. 1. Expression of mRNA for α1 chains of collagens by FMC2 cells at different passage. Cells at different passage number (P) were thawed, grown to confluence, split 1:5, and grown to approximately 85-90% confluence. Total cellular RNA was then harvested by centrifugation of cell lysates over a $CsCl_2$ cushion and subjected to denaturing electrophoresis before transfer to a nylon membrane. Equivalence of loading in different lanes was ascertained by similar intensity of ethidium bromide staining of ribosomal bands. The same blot was stripped and re-probed for expression of mRNA for the α1 chain of types I, III and IV collagen and for 28 S rRNA. Similar results were obtained in three additional experiments.

When the blots were evaluated for expression of collagenases and their inhibitors, a strikingly different pattern was observed (Fig. 2). Fibroblast-type interstitial collagenase (MMP-1) showed an intense signal at P8 that decreased 10-fold at P11, and was virtually nonexistent by P14. A strong signal for gelatinase A (MMP-2) decreased 5-fold between passages 11 and 14 (Fig. 2). There was no signal detected for gelatinase B (MMP-9) at any passage. mRNA for TIMP-1 showed a decrease similar to that seen with gelatinase A. In contrast, the total signal for TIMP-2 remained unchanged. However, there was a switch in the relative intensity of the two species of TIMP-2 mRNA, with the larger, 3.5 kb species showing 5-fold predominance at early passage and the smaller, 1.0 kb species showing 5-fold predominance by P14. These findings indicate that expression of mRNA for collagenases decreases with serial passage, opposite to the increase in expression of certain collagen mRNAs. The changes observed with collagenase inhibitors are more complex.

Changes in mRNAs related to laminin synthesis and degradation

No signal was detected at any passage for laminin α1 chain,

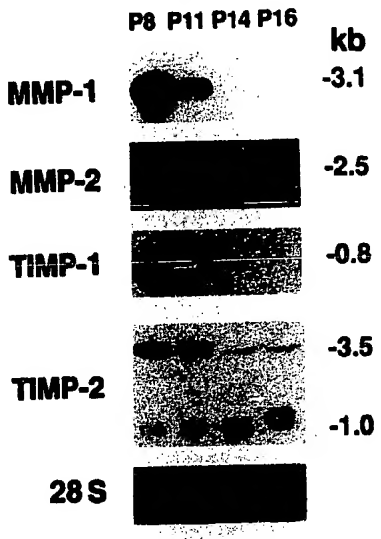

Fig. 2. Expression of mRNA for metalloproteinases and metalloproteinase inhibitors at different passages. RNA was obtained as described in the legend for Fig. 1. MMP-1, fibroblast-type interstitial collagenase; MMP-2, gelatinase A; TIMP, tissue inhibitor of metalloproteinases. A very faint signal for stromelysin-1 was detected with similar intensities at all passages, and no signal was detected for gelatinase B (MMP-9) at any passage. Similar results were obtained in 4 additional experiments.

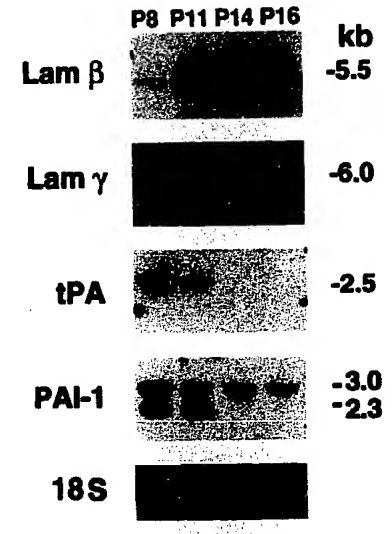

Fig. 3. Expression of mRNA for laminin and plasminogen activators. The same and similar blots to those shown in Figs 1 and 2 were stripped and re-probed for laminin α1, laminin β1, and laminin γ1, as well as for plasminogen activators and plasminogen activator inhibitors. tPA, tissue-type plasminogen activator; PAI-1, plasminogen activator inhibitor 1. No signal was detected for laminin α1, uPA or PAI-2.

consistent with a recent report that the laminin α chain expressed in mature, normal kidneys is distinct from the EHS laminin α (Lindblom et al., 1994) from which our cDNA was derived. However, laminin β1 showed a 4-fold increase from P8 to P11 and laminin γ1 increased 2-fold from P8 through P16 (Fig. 3). In contrast, tPA showed a 3-fold decrease from P8 to P11 and virtually disappeared thereafter. No signal was

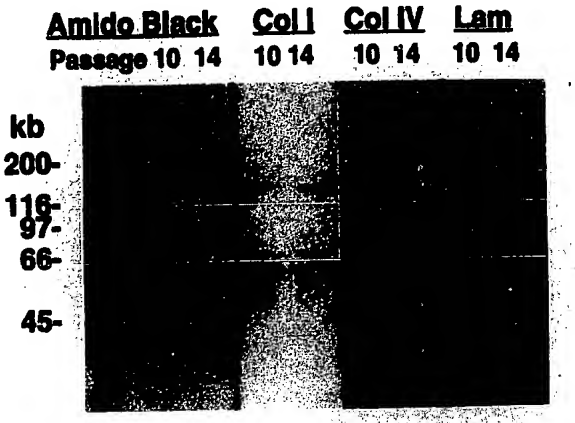

Fig. 4. Western blot for ECM proteins in medium conditioned by P10 or P14 FMC2 cells. Cultures were seeded at similar densities and grown for 3 days; at this time they appeared to have similar degrees of confluence. The culture medium was aspirated and replaced with identical amounts of medium containing no serum but otherwise identical to standard culture medium. After 24 hours, the conditioned medium was harvested and 50 μl/lane was subjected to SDS-PAGE under denaturing conditions. The supernatant fluids were run in quadruplicate pairs and each pair was stained, respectively, with amido black (to show total protein) or with antibodies to collagen I, collagen IV, or laminin. After electrophoretic transfer to Immobilon-P membranes, the blots were developed using the indicated polyclonal antisera as described in Materials and Methods. Similar results were obtained in three separate experiments.

detected for uPA at any passage. There was a faint, unchanging signal for stromelysin-1 detected at all passages (data not shown). The plasminogen activator inhibitor PAI-1 is normally expressed as two mRNA species, of 3.0 and 2.3 kb; the difference between the two transcripts is felt to reflect shorter 3′ untranslated sequence (Ny et al., 1986). Signal for the larger transcript remained essentially unchanged, with a slight decrease at P16. In contrast, the smaller transcript was not detected after P11. In general, FMC2 cells show a pattern of expression relevant to laminin turnover similar to that observed with collagen turnover, with increased expression of matrix protein and decreased expression of ECM protease mRNAs.

Production of ECM proteins by cultured FMC2 cells

Since the cells express increased amounts of mRNA for collagen IV and laminin and decreased amounts of mRNA for ECM proteases, they were examined for accumulation of ECM proteins. Media conditioned by FMC2 cells at P10 or at P14 were evaluated by immunoblot for the presence of secreted collagens and laminin (Fig. 4). Amido black staining revealed a major band of protein at about 66 kDa, probably representing residual albumin from the initial, serum-containing medium. Staining with antibody to collagen I revealed little change in expression between passages 10 and 14. There was, however, a marked increase in expression of both collagen IV and laminin. When the cells were cultured for 3 days and then stained for type IV collagen, some heterogeneity was observed (Fig. 5). However, in general the intensity of staining increased markedly as the number of passages increased. In some cases, increased punctate staining was noted extracellularly, although it is difficult to appreciate this finding from photographs due to the increased brightness of the cellular staining. These data

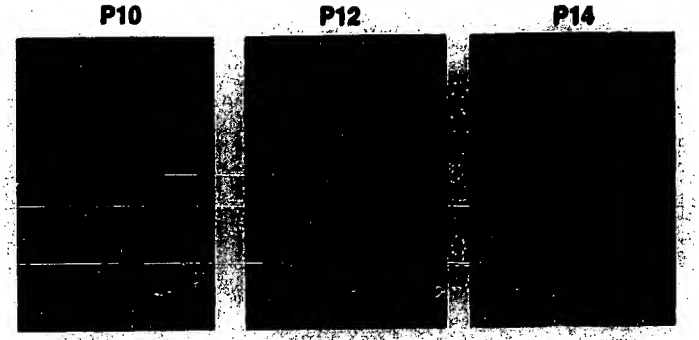

Fig. 5. Expression of type IV collagen by cells of different passage. Immunocytochemistry was performed by indirect immunofluorescence. The cells were plated on glass slides and cultured for 3 days before staining was performed as described in Materials and Methods. Representative views that include the degree of heterogeneity observed are shown in the figure. ×150.

indicate that, consistent with the northern analysis data, certain ECM proteins may accumulate in cultures of FMC2 cells subjected to an extended series of passages in culture.

Secretion of MMP activity into cell cultures

The increased amount of matrix protein could result entirely from an increase in steady-state expression of ECM protein mRNA. However, decreased ECM protease secretion might also contribute to matrix accumulation. To investigate this possibility, gelatin zymography was performed on conditioned media. Both the proenzyme (72-kDa) and activated (68-kDa) forms of gelatinase A activity were present in medium conditioned by P10 cells, but little activity was detected in medium conditioned by P14 cells (Fig. 6). Since interstitial collagenase is much less active than gelatinases in gelatin zymograms, the conditioned media were concentrated 10-fold and again subjected to zymography. Bands of gelatinolytic activity were observed at 50-55 kDa, the size of MMP-1, in P10-conditioned medium. These were not seen in P14-conditioned medium (data not shown). Thus, protease activity in the medium decreases with serial passage, consistent with changes observed in mRNA expression.

Expression of TGF-β1 mRNA by cultured cells

Because TGF-β1 has been found to play a role in several models of matrix accumulation, the cultured cells were evaluated for expression of TGF-β1 mRNA during the time at which the greatest changes in mRNA expression occur. A slight increase in TGF-β1 mRNA was seen from P8 to P14, but a much greater increase (5-fold) occurred by P16 (Fig. 7). In other experiments, the greatest increase was apparent by P14 (data not shown). These results are consistent with an increase in production of TGF-β1 with serial passage.

Effect of exogenous TGF-β1 on expression of mRNA for type I collagen, gelatinase A and TIMP-2

Since this last finding suggested that TGF-β could contribute to the phenotypic changes observed with serial passage, we tested the effects of exogenous TGF-β1 on several of the ECM components described above. Cells at P9 or P10, just before the most striking phenotypic changes occur, were cultured with graded doses of active TGF-β1 for 3 days. There was an

Fig. 6. Gelatin substrate gel chromatography of conditioned media from FMC2 cultures. The indicated amounts of medium were subjected to zymography as described in Materials and Methods. Although no signal was seen at any other molecular size under these conditions, when the media were concentrated 10-fold, a faint band was seen at 55 kDa with medium conditioned by P10 cells but not by P14 cells (data not shown). Similar results were obtained in 4 separate experiments.

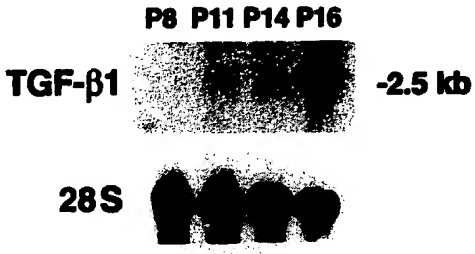

Fig. 7. Expression of mRNA for TGF-β1 at different passages. Similar results were obtained in 6 different experiments using fetal and adult cells of differing passages.

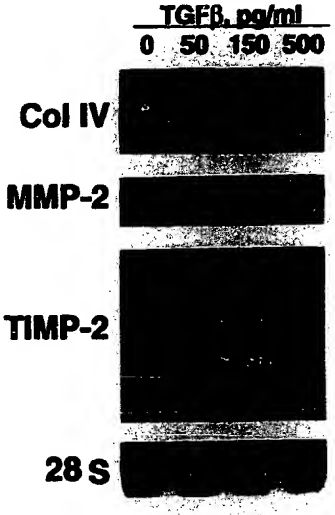

Fig. 8. Effect of TGF-β1 on expression of mRNA for collagen IV, gelatinase A and TIMP-2 mRNA. P9 FMC2 cells were treated with the indicated dose of TGF-β1 for 72 hours. To be certain that all cultures were fed and exposed to the indicated amounts of TGF-β, the medium was replaced with fresh, TGF-β-containing medium after 2 days. Total cellular RNA was harvested as described in Materials and Methods. The same blot was probed for α1(IV) collagen, gelatinase A and TIMP-2. Similar results were obtained in four additional experiments, two using cells at P9 and one each using cells at P7 and P10, as well as with mesangial cells derived from adult tissue and tested at different passages.

increase in expression of collagen α1(IV) mRNA (Fig. 8), although the extent of this increase varied in different experiments. In contrast to the findings after a prolonged series of

passages in culture, steady-state expression of mRNA for gelatinase A doubled. Moreover, TGF-β1 did not induce a change in the ratio of signal intensity comparing the two species of TIMP-2 mRNA. These results are consistent with previous reports of TGF-β-induced changes in mesangial cell mRNA expression, but are different from the changes observed in our model. In other experiments, we found that the intensity of signal for all of these mRNAs was maximal at 0.5-1.0 ng/ml TGF-β1 and decreased somewhat as the concentration of TGF-β1 approached 5 ng/ml (data not shown). Further, these results were consistently observed with cells at earlier passages as well as passages as late as P10, and with mesangial cells of either fetal or adult origin (data not shown). These findings, along with the observation that TGF-β1 mRNA increases at a later passage than does the switch in mRNAs related to ECM turnover, indicate that exogenous TGF-β1 does not cause the type of changes in cell phenotype that we have described with serial passage.

Table 1. Changes in mRNA expression with serial passage of FMC2 cells

mRNA	Change
Collagen	
α1(I)	—
α1(III)	↑↑
α1(IV)	↑↑
Laminin	
α1	Not detected
β1	↑
γ1	Slightly ↑
MMPs and TIMPs	
Interstitial collagenase (MMP-1)	↓↓↓
Gelatinase A (MMP-2)	↓↓↓
Stromelysin-1 (MMP-3)	Faint signal; no change with passage
TIMP-1	↓↓
TIMP-2	Switch in ratio of signal intensity comparing the two mRNA species
TIMP-3	Not detected
PAs and PAIs	
tPA	↓↓
uPA	Not detected
PAI-1	Decrease in smaller mRNA species
PAI-2	Not detected
TGF-β1	↑↑

DISCUSSION

Glomerulosclerosis is characterized by excessive accumulation of extracellular matrix. This process may involve deposition of abnormal matrix components, but studies have clearly indicated that additional amounts of classical components also accumulate. Thus, the net turnover of ECM in the glomerulus is altered, reflecting either increased synthesis, decreased removal, or both. Studies by various investigators have implicated both possibilities in glomerulosclerosis (reviewed by Schnaper, 1995). We subjected human fetal glomerular mesangial cells to a prolonged series of passages in culture in order to develop a model for mesangial regulation of matrix turnover. Beginning after P8, a comprehensive pattern of changes was noted, consistent with matrix accumulation (Table 1). Cells showed increased steady-state expression of mRNA for the α1 chains of types III and IV collagen and for laminin β1 and γ1. At the same or earlier passages, expression of certain ECM proteases showed a decrease. There also were marked changes in expression of two ECM protease inhibitor mRNA species, most notably a reversal of the ratio of signal intensity for the two TIMP-2 species. Concomitantly, the amount of type IV collagen and laminin present in serum-free medium conditioned by the cells for 24 hours increased, while the amount of interstitial collagenase (MMP-1) and gelatinase A (MMP-2) decreased. Cellular staining for collagen IV also increased. Thus, after a prolonged series of passages in culture, FMC2 cells manifest a matrix-accumulating phenotype, with increased synthesis and decreased degradation of ECM.

Somewhat surprising was our observation that protease inhibitor mRNA expression remained the same or even decreased with serial passage. We have not, however, excluded the possibility of post-transcriptional regulation of these proteins. Further, we have not yet addressed the importance of changes in the relative expression of the two species of TIMP-2 mRNA. The species are reported to have similar coding regions but the larger species appears to have a long 3′ untranslated region; the effect of this difference on translation into protein is the subject of ongoing investigation in several laboratories. The direct impact of the changes we have observed in protease inhibitor mRNA expression on control of ECM turnover is unclear. In the absence of significant amounts of MMPs, for example, changes in concentration of TIMPs may have little impact on proteolytic activity. However, TIMPs recently have been suggested to have additional biological roles (Murphy et al., 1993) that could affect mesangial cell function in other ways. Regardless of the significance of the changes in ECM protease inhibitor mRNA expression, these findings provides strong evidence of altered regulation of gene transcription or mRNA processing with serial passage.

The observed changes in cell phenotype could be a model for aging. In human skin fibroblasts cultured from subjects of various ages, fetal cells at P20 showed similar characteristics to earlier passages of fibroblasts from 80-year-old donors (Takeda et al., 1992). Glomerulosclerosis is a common histological finding with aging in vivo both in humans (Kaplan et al., 1975) and in animals (Couser and Stilmant, 1975). Despite this 'aging process,' FMC2 cells at P14 to P16 do not appear to be typically senescent in that they continue to produce proteins and show stable growth rates. Indeed, they remain viable through P25. Further, there is no general defect in mRNA or protein synthesis at P14; the fact that various mRNAs and proteins increase, remain stable or decrease in expression indicates that the cells' synthetic machinery remains intact. The findings therefore represent specific differences between different cell passages in the regulation of expression of specific proteins. Indeed, it has been suggested that changes in patterns of matrix production represent the degree of differentiation or de-differentiation of cells (Foidart et al., 1980); our model could therefore represent an aspect of a maturation process unique to fetal (as opposed to adult) cells.

Previous studies examining other cell types have identified changes in cellular production of matrix proteins or ECM proteases with serial passage in culture that, while relevant to the present series of experiments, show significant differences. First, collagen gene expression does not invariably increase with serial passage. For example, expression of types I and III

collagen was observed to decrease with serial passage in human skin fibroblasts (Takeda et al., 1992). Second, MMP production increased with passage of retinal pigment epithelium, articular chondrocytes and corneal fibroblasts (Alexander et al., 1990; Fini and Girard, 1990; Lefebvre et al., 1991). In contrast, our experiments with FMC2 cells show stable collagen I production, increased collagen IV synthesis and decreased MMP production. The difference between our model and other reports of changes in MMP expression with serial passage are consistent with the observation that mesangial cell production of gelatinase A is regulated in a unique manner compared with other cells (Marti et al., 1994). To the extent that changes with serial passage represent a model of cell 'aging,' these differences suggest distinct aspects of the mesangial cell response to aging. Therefore, our findings could explain why the glomerulus appears to be particularly susceptible to the sclerotic process as individuals grow older.

FMC2 cells at later passages (P14 to P16 in different experiments) produced large amounts of TGF-β1 mRNA. Although it is possible that increased TGF-β1 mRNA expression does not lead to equally increased protein production, TGF-β has been associated with mesangial expansion. Therefore, we used graded doses of TGF-β1 to treat mesangial cells at a point just prior to the phenotypic switch we have observed. This treatment did cause some increase in expression of mRNA for type IV collagen, but it did not change the relative signal intensity for the larger of the TIMP-2 mRNA species and, consistent with observations of others investigating mesangial cells (Marti et al., 1994), TGF-β treatment actually increased gelatinase A mRNA expression. Thus, the phenotypic switch we have observed is not produced by adding TGF-β1 to cultures. This result is consistent with a recent preliminary report (De Heer et al., 1994) indicating that TGF-β1 production accompanies development of glomerulosclerosis but is not by itself sufficient to directly cause the lesion. However, from the present series of experiments, we cannot rule out the possibility that endogenous TGF-β (produced by the cells) participates in the switch to a matrix-accumulating phenotype.

Since cell culture was initiated from a primary isolate of glomerular mesangial cells, it is possible that two subpopulations of mesangial cells were present in the initial culture, and that one showed a decrease in growth at P8-P10 while the other grew only slowly until the same passage. This is not likely, since maintenance of a stable growth rate (calculated time for doubling of cell number in culture, 55-60 hours) within the entire culture would have required a series of remarkably coordinated changes in the growth rates of each population. Nonetheless, the possibility that the classical and matrix-accumulating phenotypes represent two different populations of cells, or that a significant minority population arose and altered regulation of matrix turnover in the rest of the cells, cannot be formally ruled out by the present series of experiments.

The difference in gene expression between earlier- and later-passage FMC2 cells has been observed six different times when the cells were subjected to serial passage. Although the cause of the observed phenotypic 'switch' is uncertain, the two phenotypes clearly define contrasting classical and matrix-accumulating populations of cultured mesangial cells. In view of the consistency with which this phenotypic switch is observed, and the fact that the pattern of differences observed is unique among published reports of such changes in serially-passaged cells, our findings suggest the potential for further insight into regulation of matrix turnover in glomerular mesangial cells from studying the FMC2 cells. Because the later-passage cells appear to be accumulating matrix, comparison of the two phenotypes may permit determination of events relevant to matrix accumulation in vivo. Thus, our findings may serve as a model for cellular events involved in the pathogenesis of glomerulosclerosis.

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CS Renal Unit, Fundacion Jimenez Diaz, Universidad Autonoma, Madrid, Spain.
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Transforming Growth Factor-β1 Up-regulates Type IV Collagenase Expression in Cultured Human Keratinocytes*

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During the wound healing process lysis of basement membranes precedes keratinocyte migration into the wound bed. We studied, *in vitro*, whether this degradation of basement membranes could be regulated by transforming growth factor-β1 (TGF-β1), which is known to accelerate wound healing *in vivo*. Transforming growth factor-β1 was found to increase the expression of both 92- and 72-kDa type IV collagenases (gelatinases) in cultured human mucosal and dermal keratinocytes. The 92-kDa enzyme predominated in both unstimulated and stimulated cultures. The 92-kDa form was stimulated over 5-fold, and the other form by a factor of 2–3. This increase in the synthesis of type IV collagenases was associated with a marked increase in the mRNA levels of these enzymes as well. The induction of the 92-kDa enzyme was similar in culture medium containing either 0.15 or 1.2 mM calcium chloride. Rat mucosal keratinocytes secreted only 92-kDa type IV collagenase, the secretion of which was not regulated by TGF-β1. Also, TGF-β1 did not cause any significant induction (maximum about 1.2-fold) of either type IV collagenase in human gingival fibroblasts. The induction levels of both collagenases in human keratinocytes were independent of the type of the extracellular matrix the cells were grown on. However, the basement membrane matrix (Matrigel) activated about half of the 92-kDa type to its 84-kDa active form. The data suggest that TGF-β1 has a specific function in up-regulating the expression of type IV collagenases in human keratinocytes, offering a possible explanation of how keratinocytes detach from basement membranes prior to the migration over the wound bed.

Matrix metalloproteinases (MMPs)[1] compose a family of at least eight structurally and functionally homologous extracellular enzymes, which have a major role in the degradation of connective tissue components (see Refs. 1–4 for reviews). The MMP family includes two interstitial collagenases (fibroblast-type and polymorphonuclear-type), three stromelysins (1, 2, and 3), a small putative uterus metalloprotease (PUMP-1), and two type IV collagenases (gelatinases) in 72- and 92-kDa forms. These enzymes are believed to be important during normal physiological remodeling of the matrix and in pathological processes such as embryogenesis, wound healing, inflammation, tumor invasion, and metastasis (4–6). The primary structure of several matrix metalloproteinases has been recently obtained through amino acid sequencing and by sequencing the isolated cDNA clones (3). These enzymes share a high degree of structural homology, including a conserved sequence at the Zn-binding site which is a part of the active center of these enzymes (7, 8). They are all secreted in a latent form which may be self-activated or activated proteolytically or chemically, for example by plasmin, trypsin, or organomercurials. Tissue inhibitor for metalloproteinases (TIMP; 9) is a specific physiological inhibitor for MMPs.

Most of the MMPs cleave several different matrix components and have overlapping substrate specificities. The two type IV collagenases, the 72- and 92-kDa forms, degrade basement membrane collagen in a specific site (7, 10–12). They are both able to degrade also types V (7, 8, 12, 13), VII (7, 14), and X collagens (15), as well as gelatin (7, 12, 13, 16). The biological function of type IV collagenases has not been fully established, although they have been shown to be linked to metastatic growth, by facilitating the penetration of basement membranes by tumor cells (5). The production of 72-kDa type IV collagenase (13, 17–19) and 92-kDa form (8, 12, 16) by normal cells suggests an important role in physiological processes as well. However, the regulation of their expression and activity in different normal cell lines is still not understood.

Transforming growth factor-β1 (TGF-β1) is a multifunctional, hormone-like peptide that controls cell proliferation and differentiation in several cell lines (20–22). It is probably an important mediator also in embryogenesis, wound healing, and bone remodeling. Many cells synthesize TGF-β1 and have a specific receptor for this peptide. Effects of TGF-β1 are cell-specific. For example, TGF-β1 acts mitogenically for fibroblasts by inducing the synthesis of c-*sis* protooncogene and platelet-derived growth factor-like material (23), while it reversibly inhibits the proliferation of keratinocytes and endothelial cells (24, 26) via the suppression of c-*myc* gene transcription (28) or via dephosphorylation of retinoblastoma gene product (27). However, several squamous carcinoma cell lines have lost the normal inhibitory response to TGF-β1 (30). Fibroblast matrix production is enhanced by TGF-β1 both *in vivo* and *in vitro*. In animal experiments, TGF-β1 stimulates wound healing processes (20, 29, 30). *In vitro* TGF-β1 has

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[1] The abbreviations used are: MMP, matrix metalloproteinase; TGF-β1, transforming growth factor-β1; KBM, keratinocyte basal medium; SDS, sodium dodecyl sulfate; TIMP, tissue inhibitor of metalloproteinase; TPA, tetradecanoyl phorbol acetate; PBS, Dulbecco's phosphate buffered saline; GAPHD, glyceraldehyde-3-phosphate dehydrogenase; bp, base pair(s).

been shown to increase the synthesis of the major stromal components such as fibronectin (31–33), type I collagen (31), and proteoglycan (36) in mesenchymal and epithelial cells. One of the mechanisms of matrix accumulation by TGF-β1 is its inhibitory action on matrix-degrading enzymes. It has been shown to inhibit the expression of plasminogen activators in human skin fibroblasts (35). Furthermore, TGF-β1 inhibits in fibroblasts the induction of interstitial collagenase (36) and stromelysin caused by other growth factors, most likely through a *fos*-binding sequence (37). In addition, TGF-β1 enhances the production of TIMP (18, 36) and plasminogen activator inhibitor (35, 38).

Of the MMPs, human keratinocytes have been shown to produce interstitial collagenase (39) and 92-kDa type IV collagenases (8), but not stromelysin (8). The regulation of type IV collagenases in keratinocytes has not been studied in detail. We report here that human mucosal keratinocytes produce elevated levels of 92- and also 72-kDa type IV collagenases in response to TGF-β1, and suggest that this response may be important to the wound healing process by facilitating the detachment of epithelial cells from the basement membrane prior to migration across the wound bed.

EXPERIMENTAL PROCEDURES

Cell Culture—Four normal human mucosal keratinocyte cell lines were isolated from surgical gingival biopsies by explant culture technique and were cultured in serum-free low calcium KBM (Clonetics Corp., San Diego, CA) supplemented with epidermal growth factor, hydrocortisone, insulin, bovine pituitary extract, antibiotics, and antimycotic (200 IU/ml penicillin, 50 μg/ml gentamycin, and 2.5 μg/ml Fungizone; Clonetics Corp.). In some experiments low calcium medium (0.15 mM CaCl) was changed to high calcium medium (1.2 mM CaCl) by adding 200 mM calcium chloride solution. Human epidermal keratinocytes were purchased from Clonetics Corp. An established rat mucosal keratinocyte cell line (40) was cultured in Ham's F-12:Dulbecco's modified Eagle's media (1:1), supplemented with insulin (5 μg/ml), transferrin (1 μg/ml), and bovine serum albumin (5 mg/ml) as described for mammary carcinoma cells by Stevenson *et al.* (41). For measuring gelatinolytic activities, and isolating total cellular RNA, human mucosal keratinocytes (between passages two and six) were cultured to about 60% confluence and were treated with TGF-β1 (0–4 ng/ml; R&D Systems Inc., Minneapolis, MN) or TPA (10^{-7} M; Sigma) for 12 or 24 h. Normal human gingival fibroblasts were cultured from biopsies of healthy gingiva taken during the deliberation surgery of unerupted maxillary canines. Fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 1 mM glutamine, 1 mM sodium pyruvate, antibiotics (50 μg/ml streptomycin, 50 IU/ml penicillin), and 10% heat-inactivated fetal calf serum. For the TGF-β1 treatments the subconfluent cell cultures were washed several times with PBS to remove traces of serum and incubated with serum-free medium with or without TGF-β1 (4 ng/ml) for 24 h. Fibroblasts used in the assays were between passages four and nine. Cell numbers were measured by staining fixed and air-dried cells with 1% crystal violet in 0.1 M boric acid, pH 6.0, for 20 min (42). Unbound stain was removed by rinsing with distilled water, and the cells were air-dried again. The stain was then extracted with 10% acetic acid and measured spectrophotometrically at 595 nm.

Substrate Assays—For studying the effects of TGF-β1 on type IV collagenases the keratinocytes were cultured to subconfluency on different substrates (tissue culture plastic, fibronectin, laminin, type I collagen, and basement membrane) after which the medium was changed and replaced with media containing TGF-β1 (2 ng/ml), TPA (10^{-7} M) or control medium for 24 h. The media were collected and analyzed by zymography. The cell culture wells were coated with 50 μg/ml of fibronectin or laminin (gifts from Dr. Kenneth Yamada, Laboratory of Developmental Biology, National Institute of Dental Research, Bethesda, MD) in PBS in the presence of Ca^{2+} and Mg^{2+}, and then countercoated with 10 mg/ml bovine serum albumin (Sigma) in PBS for 30 min. Type I collagen substrate, prepared from Vitrogen 100 (Collagen Corp., Palo Alto, CA), and basement membrane, prepared from Matrigel (Collaborative Research), were used essentially as described by the manufacturers, except that the basement membrane matrix gel was dried overnight before use.

Zymography—For zymography, the method of Heussen and Dowdle (43) was used, except that the substrate was gelatin which had been labeled fluorescently with 2-methoxy-2,4-diphenyl-3[2H]furanone (Fluka, Ronkonkoma, NY) by the method of O'Grady *et al.* (44). The advantage of this method is that the lysis can be monitored visually, or by photographic inspection under long-wave UV light, and the capacity of proteins in the samples to bind Coomassie Brilliant Blue does not affect the pattern of lysis. Samples for zymography were preincubated for 1 h at 22 °C with 1% SDS and electrophoretic sample buffer. Prestained standard proteins for M_r determination were reduced with 5% 2-mercaptoethanol prior running the gel. Zymography was performed in 0.75–1.5-mm 10% polyacrylamide slab gels containing 1–2 mg/ml gelatin. The gels were incubated at 37 °C and periodically monitored for lysis by long-wave UV illumination, after which they were washed with water at 22 °C for 20 min and stained in 0.2% Coomassie Brilliant Blue R250 (Kodak) in 30% methanol, 7.5% acetic acid. The gels were destained in 30% methanol, 7.5% acetic acid and dried. Cleavage rate estimates were obtained by determining the rates of disappearance of the gelatin by scanning the Coomassie Blue-stained gels with a computing densitometer model 300A (Molecular Dynamics, Santa Ana, CA). In a set of experiments zymograms were incubated for 4 h at 37 °C with control buffer, 25 mM EDTA, 1 mM 1,10-phenanthroline, or 2 μg/ml recombinant human TIMP, a gift from Dr. David Carmichael (Synergen, Boulder, CO). The relationship between the enzyme concentration and the scanned zymogram peak height was tested and found to be essentially linear.

Preparation of Labeled cDNA Probes—The cDNA probes used in this study were: pHcGAP, a 1200-bp clone that codes for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; American Type Culture Collection, probe 57091); pK121, a 2181-bp clone that codes for human 72-kDa type IV collagenase (gelatinase) (45); p92174.1, a 2100-bp clone that codes for human 92-kDa type IV collagenase (gelatinase) (8). For Northern and *in situ* agarose hybridizations the clones were labeled by nick-translation with [^{32}P]dCTP (Amersham Corp.).

Assays of mRNAs—Total cellular RNA was extracted and purified according to the method described by Cromczynski and Sacchi (46). For Northern analysis equal amounts (10 μg) of the RNA samples were fractionated on 0.7% agarose, 18% formaldehyde gels and transferred to nylon filter (Zeta-Probe, Bio-Rad). Hybridization was carried out at 42 °C for 24 h in a solution containing 50% (v/v) formamide, 0.25 M $NaHPO_4$, 0.25 M NaCl, 7% (w/v) SDS, 1 mM EDTA, and labeled cDNA clone as recommended for Zeta-Probe. The filters were washed twice for 15 min at room temperature in a solution containing 2 × SSC (SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7), 0.1% SDS, and 0.5 × SSC, 0.1% SDS, respectively, and once at 65 °C in a solution of 0.1 × SSC, 0.1% SDS. In some experiments the *in situ* agarose gel hybridization method described by Ahmad *et al.* (47) was used in RNA analysis. The gel or the filter were exposed to Kodak X-Omat film for 18–48 h with an intensifying screen at −70 °C.

RESULTS

Zymographic Analysis of 72- and 92-kDa Type IV Collagenases—Several human mucosal keratinocyte and fibroblast cell lines together with one human epidermal keratinocyte cell line were tested for the production of the two forms of type IV collagenase (72- and 92-kDa enzymes). All cell lines of epithelial origin expressed both the enzymes at about a 1:2 ratio while fibroblasts synthesized predominantly the 72-kDa type IV collagenase (Fig. 1). In some experiments we could also detect a high molecular mass enzyme (molecular mass > 200 kDa). All of the proteinase activities that were detected in gelatin zymograms were matrix metalloproteinases since they were inhibited by 1,10-phenanthroline, EDTA, and TIMP (not shown).

Effects of TGF-β1 on the 92-kDa Type IV Collagenase—Treatment of human keratinocytes by TGF-β1 caused a dose-dependent induction of 92-kDa type IV collagenase. Maximal stimulation was obtained by 2 ng/ml dose of the growth factor (Fig. 2). Although there were some differences between cell lines in the amount of enzyme secreted, more than 5-fold

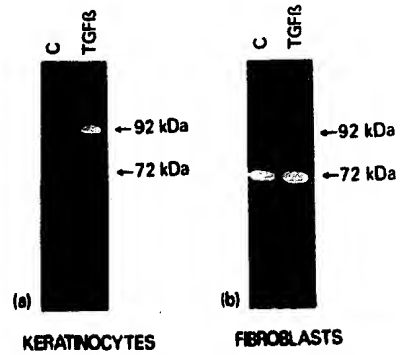

FIG. 1. **Comparison of the effects of TGF-β1 on human mucosal keratinocytes (*a*) and fibroblasts (*b*).** Human mucosal keratinocytes were grown in KBM medium, supplemented with growth factors and antibiotics to about 60% confluence, and incubated for 24 h with 2 ng/ml TGF-β1 or without (*C*). Human gingival fibroblasts were cultured to subconfluence in 10% fetal calf serum containing Dulbecco's modified Eagle's medium, and then placed into serum-free medium with 2 ng/ml TGF-β1 for 24 h or without (*C*). From both cultures media samples of 35 μl were collected and analyzed by zymography. *Arrows* indicate the positions of 72- and 92-kDa type IV collagenases.

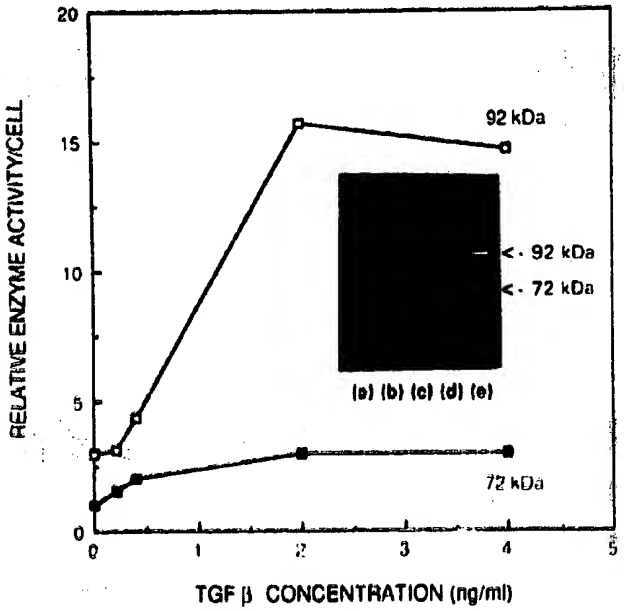

FIG. 2. **Effect of TGF-β1 on the activity of 72- and 92-kDa type IV collagenases in human mucosal keratinocytes.** Human mucosal keratinocytes were treated with different concentrations of TGF-β1 for 24 h after which the media were analyzed for enzyme activities using 10% polyacrylamide gels containing 2 mg/ml fluorescently labeled gelatin. The fluorescent zymograms were monitored for 18-h incubation at 37 °C. At the conclusion of the incubation the gels were stained with Coomassie Brilliant Blue R250 and scanned by densitometry. The cell numbers were measured by the crystal violet staining method. The densitometric scans were then corrected with the cell numbers. The values represent relative enzyme activities of 72-kDa (■) and 92-kDa (□) per cell. *Inset* shows zymogram of human keratinocyte culture medium after the treatment with 0, 0.2, 0.4, 2, or 4 ng/ml of TGF-β1 (*a*, *b*, *c*, *d*, *e*, respectively). The *arrows* indicate the position of 72- and 92-kDa type IV collagenases.

stimulation was observed with all four mucosal and one epidermal keratinocyte cell lines tested. Treatments were routinely performed in low calcium keratinocyte growth medium containing all necessary supplement including epidermal growth factor and insulin to promote cell proliferation. To investigate whether the observations made were caused by complicated interactions between TGF-β1 and other growth factors, a set of experiments were performed in basal keratinocyte growth medium containing only antibiotics. The results from these control experiments were similar to those obtained from experiments with complete growth medium (not shown). Increasing the extracellular calcium concentration (from 0.15 to 1.2 mM) of keratinocyte cultures promoted terminal differentiation as measured by increased cell clustering and expression of the involucrin gene (not shown). Simultaneous treatment with 1.2 mM calcium and TGF-β1 for up to 24 h caused similar induction of the 92-kDa enzyme as seen in cultures maintained in low calcium conditions (Table I). A rat mucosal keratinocyte cell line also expressed a homologue of the 92-kDa enzyme as their major type IV collagenase. However, it was not regulated by TGF-β1 (Table I). Human gingival fibroblast 92-kDa type IV collagenase expression was stimulated only about 1.2-fold by TGF-β1 (Table I and Fig. 1).

In order to determine that there was an increase in the mRNA abundance of the 92-kDa type IV collagenase in human keratinocytes exposed to TGF-β1, Northern blot experiments were performed. Cells were treated with 2 ng/ml of TGF-β1 for 12–24 h after which media were collected for zymographic analysis of the type IV collagenase, and the cells were used for isolation of the total cellular mRNA. A clear induction of both the protein and the coding mRNA was observed after 12 h of incubation (Fig. 3). Progressive stimu-

TABLE I

Stimulation of type IV collagenases by TGF-β1

Keratinocyte and fibroblast cell cultures were developed and maintained as described under "Materials and Methods." Stimulation of type IV collagenases was measured in serum-free media supplemented with 2 ng/ml of TGF-β1 for 24 h by zymography.

	92 kDa	72 kDa
Human mucosal keratinocytes[a]	+++	+
Human epidermal keratinocytes[a]	+++	+
Rat mucosal keratinocytes	−	0
Human gingival fibroblasts	±	±

[a] The stimulation of the enzymes were analyzed both in the KBM containing either 0.15 mM or 1.2 mM calcium chloride solution.

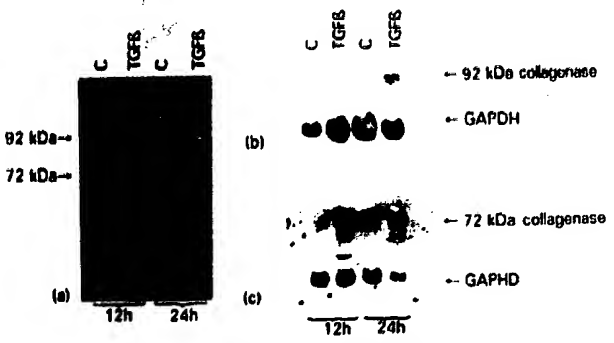

FIG. 3. **Stimulation of mRNA levels of 92- and 72-kDa type IV collagenases following treatment with TGF-β1.** Human mucosal keratinocytes were grown to about 60% confluence in KBM medium and were incubated with or without 2 ng/ml TGF-β1. After either 12 or 24 h, medium was collected and analyzed by zymography (*a*). Total cellular RNA was extracted and electrophoresed on 0.7% agarose/formaldehyde gels and transferred onto Zeta-Probe nylon membrane. The resulting blots were hybridized to the 92-kDa type IV collagenase and GAPHD probes that had been ^{32}P-labeled by nick-translation (*b*). *In situ* Northern technique described by Ahmand *et al.* (47) was used to detect the 72-kDa type IV collagenase, where approximately 10 μg of total RNA was electrophoresed on 0.7% agarose/formaldehyde gel, and after drying the gel was hybridized to nick-translated, ^{32}P-labeled 72-kDa type IV collagenase and GAPDH cDNA probes.

lation of 92-kDa type IV collagenase was seen with increasing the incubation time up to 24 h. In fact, at both time points the mRNA levels of the 92-kDa enzyme in control culture was almost undetectable.

We then studied whether the induction of the 92-kDa type IV collagenase in human keratinocytes by TGF-β1 is dependent on the extracellular signalling by matrix with which the cells are in contact. Cells were cultured on tissue culture plastic, fibronectin, type I collagen, laminin, and a basement membrane substratum, Matrigel (Fig. 4). TGF-β1 stimulated the expression of the 92-kDa type IV collagenase on all substrates. Cell cultures on all of the substrates except Matrigel, were found to contain type IV collagenase in its latent form (92 kDa). A significant proportion (about half) of the enzyme was present in its active form when keratinocytes were cultured on Matrigel (Fig. 4). Exposure of these cultures to TGF-β1 did not change this activation process. The activator of the type IV collagenase was present in the basement membrane material itself, and was subsequently found to activate the latent enzyme expressed by the keratinocytes cultured on other substrates such as tissue culture plastic or collagen (Fig. 5).

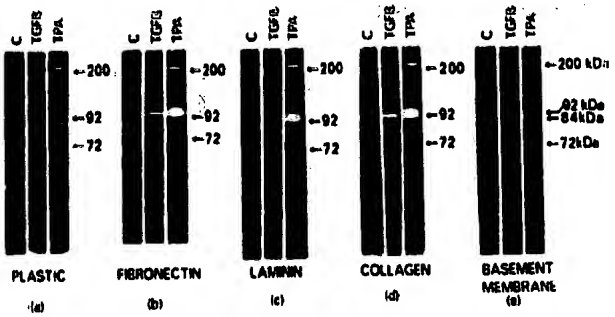

FIG. 4. **Effects of TGF-β1 and TPA on type IV collagenase in keratinocytes cultured on different matrices.** Human mucosal keratinocytes were cultured on cell culture plastic (*a*), fibronectin (*b*), laminin (*c*), type I collagen (*d*), or on the basement membrane substrate (Matrigel, *e*) in the presence of control medium (*C*), 2 ng/ml TGF-β1 (*TGFβ*), or 10^{-7} M TPA (*TPA*) for 24 h. The media samples were analyzed by fluorescent zymography. The *arrows* indicate the positions of 92-kDa type IV collagenase in a latent (*92 kDa*) and in active (*84 kDa*) form, a 72-kDa type IV collagenases, and the high molecular mass gelatinase (>200 kDa).

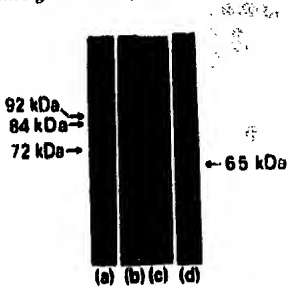

FIG. 5. **Activation of 92-kDa type IV collagenase by basement membrane substrate (Matrigel).** Human mucosal keratinocytes were cultured on a tissue culture plastic in the presence of TGF-β1 for 24 h. The media (*a*) were collected and aliquots of it were transferred to a Matrigel substrate (*b*) or to tissue culture plastic (*c*) and incubated for 24 h at 37 °C. Fresh control KBM media samples were first incubated without cells on a solid basement membrane substrate following the extraction to the electrophoresis sample buffer (*d*). The samples from all cultures were analyzed by zymography as described under "Materials and Methods." The *arrows* indicate the positions of 92-kDa type IV collagenase in a latent (*92 kDa*) and in active (*84 kDa*) form. The position of the 72-kDa enzyme, as well as that of a smaller molecular mass gelatinase (about 65 kDa) present in Matrigel are indicated.

Effects of TGF-β1 on the 72-kDa Type IV Collagenase—TGF-β1 enhanced the expression of the 72-kDa type IV collagenase in human keratinocytes by about 2–3-fold as measured from the zymograms and Northern blots (see Figs. 2 and 3). In general, in experiments the induction of the 72-kDa type IV collagenase was coordinate with but lower than the 92-kDa enzyme in the presence of TGF-β1. In human gingival fibroblasts TGF-β1 up-regulated the 72-kDa form by only 1.2-fold (Table I and Fig. 1).

Effects of TPA on 92- and 72-kDa Collagenases—Expression of the 92-kDa type IV collagenase was induced (7–8-fold) by 10^{-7} M TPA in human keratinocytes grown on all of the substrates tested (Fig. 4). This induction was thus even higher than previously seen with TGF-β1. TPA did not have any clear effect on the 72-kDa enzyme form. However, in some cultures stimulated with TGF-β1, and especially with TPA, an extra gelatinase, with a band of about 200 kDa in size, was seen in the zymograms (Fig. 4). In human gingival fibroblast cultures the expression of both 72- and 92-kDa enzymes was not regulated by TPA (not shown).

DISCUSSION

TGF-β1 is usually considered as an anabolic growth factor for fibroblasts: it reduces the production of interstitial collagenase (36) as well as stromelysin (37) and enhances the expression of the inhibitors of these enzymes such as TIMP (18, 36). In addition, the production of several matrix components, like collagen and fibronectin (21, 31), is induced by TGF-β1. Thus the accumulation of matrix components seems to be an important effect of TGF-β1 in fibroblastic cells *in vitro* (20). However, TGF-β1 can also stimulate the expression of MMPs. For example, Overall *et al.* (18) found that the expression of 72-kDa type IV collagenase by gingival fibroblasts is stimulated modestly in the presence of TGF-β1. We also tested gingival fibroblasts, but the maximal stimulation of both type IV collagenases by TGF-β1 was only about 1.2-fold, while in cultured human mucosal and dermal keratinocytes the expression of the protein of 72- and 92-kDa enzymes were about 2–3- and 5-fold, respectively. However, TGF-β1 had no effect on the expression of these enzymes in a rat mucosal keratinocyte cell line. This could be a species specific feature of TGF-β1 regulation, or related to the different phenotype of the rat cells, since they are immortal and do not require specific growth factors to support their proliferation *in vitro*. Low levels of the 92-kDa enzyme could be detected in the culture medium from fibroblasts by zymography, and the activity was unresponsive to TGF-β1. The 72-kDa enzyme is clearly the dominating type IV collagenase in human fibroblast cultures, but we were able to identify it also in human mucosal keratinocytes. Mucosal keratinocytes seem to differ from normal human bronchial epithelial cells, which have not been shown to secrete a detectable level of 72-kDa type IV collagenase (7).

In keratinocyte experiments, TPA, a known inducer of collagenase expression in a variety of cell lines, was used as a positive control substance to probe the capacity to increase the type IV collagenase expression. In the 92-kDa case, the stimulation was even stronger than for TGF-β1. In addition, TPA seemed to stimulate a noncharacterized approximately 200-kDa gelatinase. Such a large molecular mass band has also been observed in studies of neutrophil and macrophage gelatinases (16), but it is still unclear whether it represents a distinct enzyme species, a dimer of the 92-kDa enzyme, or a form of the 92-kDa enzyme which is posttranslationally modified, *e.g.* by extensive glycosylation. The difference between the stimulation of 72- and 92-kDa forms by TPA could be due

to the differences in the corresponding genes. The latter gene has in the promoter area two AP-1 binding sites[2] for nuclear transcription factor, which mediates the effect of TPA (48), while this site is not present in the 72-kDa enzyme gene (49). In addition, it is possible that TPA also induces TGF-β1 expression, thereby leading to enhanced 92-kDa enzyme production. This possibility is supported by the *in vivo* observations (50), in which TPA treatment of epidermis seemed to increase the mRNA levels of TGF-β1 in the suprabasal epidermal cells.

Extracellular matrix plays a crucial role in many cellular phenomena such as embryogenesis and differentiation. In endothelial cells, phenotypic modulation by TGF-β1 is dependent on the composition and organization of the matrix (51). In human keratinocyte cultures, however, effects of TGF-β1 on the 92-kDa type IV collagenase were similar, independent on which extracellular matrix component the keratinocytes were grown. Also the basal levels of the type IV collagenases were similar on different matrix substrates. This suggests, first, that matrix does not directly regulate the expression of type IV collagenases, and second that the effect of TGF-β1 is independent of the matrix environment. Therefore, the excess collagenase production induced by TGF-β1 is probably not caused through any indirect influence on the matrix, such as matrix accumulation via enhanced fibronectin synthesis, shown in keratinocytes (33), but rather by some more direct effect. In the study by Emonard *et al.* (52) the basement membrane substrate Matrigel was able to induce the production of interstitial as well as type IV collagenolytic activities by normal throboblastic cells. This indicates that basement membranes are able to regulate the activities of MMPs in these "invasive"-like cells. Interestingly, although we did not see any clear induction of type IV collagenases by Matrigel substrate, we found that it contained an activator for the 92-kDa enzyme, the enzyme which is usually recovered in its latent form from cells grown in all other substrata. This activator was present in the solid phase of Matrigel, and no cells were required for the activation. This activation process might have biological significance *in vivo* where basement membrane matrix could increase the degradation rate of the type IV collagen component by 92-kDa collagenase activation. It is possible that the extra small molecular weight gelatinase present in the zymography of basement membrane matrix itself (Fig. 5) could be the activator of the 92-kDa type IV collagenase.

What is the biological significance of the observed new effect of TGF-β1 on human keratinocytes? As previously shown, keratinocyte matrix fibronectin production is promoted by TGF-β1 (32), and although it has inhibitory effect on keratinocyte proliferation, it has been shown to increase their migration (32). The stimulus for keratinocyte migration by TGF-β1 could partially be affected by the increased fibronectin deposition, since fibronectin is known to enhance the migratory potential of epithelial cells (32, 53). In wounds, keratinocytes migrate laterally to cover the exposed connective tissue (54). TGF-β1 is present in high concentrations in these conditions, since it is released in large quantities by platelets from the wound bed vessels, and is thus available for keratinocytes. Lysis of the basement membrane is seen prior to the keratinocyte lateral movement to the wound bed (54). Furthermore, migratory epithelial cells have been shown to produce type IV collagenase (39). We suggest therefore, that this degradation could be caused by TGF-β1-mediated, enhanced expression of both the 92- and 72-kDa type IV collagenase which dissolve the most abundant skeletal element of the basement membrane, the type IV collagen. An important step in this cascade could be the activation of the 92-kDa enzyme form by some basement membranes components. Furthermore, type IV collagenases also degrade type VII collagen, the major structural component of the anchoring fibrils, which are critical for epidermal-dermal adhesion in the basement membrane zone.

In summary, TGF-β1 might be the key factor for all the early steps involved in wound healing. It stimulates the matrix deposition by fibroblasts; inhibits keratinocyte proliferation, but enhances their migration to cover the denuded surface. Prior to the migration process the keratinocytes have to detach from the underlying basement membrane. In this study we have shown the possible importance of TGF-β1 in facilitating the detachment process of keratinocytes; it clearly stimulates the expression of the type IV collagenases which are able to degrade the main collagen component of basement membranes.

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